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"Psychoactive substances present in Salvia divinorum acquired in smartshops or in the Internet"

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“Life is a succession of lessons which must be lived to be understood.”

Helen Keller

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Resumo

A Salvia divinorum é presentemente considerada uma das drogas recreativas mais populares entre adolescentes, sendo legalmente comercializada em vários países e regiões, em "smart shops" e através da internet. Na presente pesquisa, o composto alucinogénio da droga, a salvinorina A, foi identificado e quantificado, juntamente com outras 3 salvinorinas (B, C, e D), com o intuito de verificar a informação fornecida aos consumidores em 10 produtos contendo extratos concentrados de salvinorina A, com potências rotuladas entre "5X" e "60X". A extração com acetonitrilo revelou-se eficiente, e a análise foi realizada por cromatografia gasosa e espectrometria de massa. As concentrações de salvinorina A variaram entre 2,6 µg/mg to 521,2 µg/mg, muitas vezes contrariando a informação adiantada pelos comerciantes.

Palavras-chave: *Salvia divinorum*, salvinorina A, extratos concentrados, *Smart shops*, drogas legais.

Abstract

Nowadays, Salvia divinorum is considered one of the most popular recreational drugs among adolescents, being legally commercialized in many countries in "smart shops" and internet websites. In the present research, the hallucinogenic compound of the drug, salvinorin A, was identified and quantified, alongside with 3 other salvinorins (B, C and D), in order to verify the information provided to consumers in 10 products containing concentrated extracts of salvinorin A, with labeled potency between "5X" and "60X". The extraction was found to be efficient with acetonitrile, and the analysis was performed by gas chromatography mass spectrometry. The concentrations of salvinorin A ranged from 2,6 µg/mg to 521,2 µg/mg contradicting the information advanced by marketers.

Keywords: *Salvia divinorum*, salvinorin A, concentrated extracts, *Smart shops*, legal drugs.

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Abbreviations List

Apr: April;

Aug: August;

BNI: Binaltorphimine;

Bp: Basis Pair;

cAMP: Cyclic adenosine monophosphate;

CB_1: Cannabinoid receptor type 1;

Da: Daltons;

DESI: Desorption Electrospray Ionization;

DNA: Deoxyribonucleic acid;

DOR: Delta opioid receptors / δ opioid receptors;

EMCDAA: European Monitoring Centre for Drugs and Drug Addiction;

EMEA: European Medicines Agency;

EOM-SB: Ethoxymethyl Ether of Salvinorin B;

ESI: Electrospray Ion;

Feb: February;

FDA: Food and Drugs Administration;

G proteins: Guanosine nucleotide-binding proteins;

GC: Gas Chromatography;

HPLC: High Performance Liquid Chromatography;

INFARMED: Autoridade Nacional do Medicamento e Produtos de Saúde;

IT: Ion Trap;

IV: Intravenous;

Jun: June;

KOR: Kappa Opioid Receptors;

LC: Liquid Chromatography;

LLE: Liquid-liquid extraction;

LOD: Limit of Detection

LOQ/LLOQ: Limit of Quantification / Lower Limit of Quantification

LSD / LSD-25: Lysergic acid diethylamide;

MOR: Mu opioid receptors / μ opioid receptors;

MS: Mass Spectrometry;

m/z: mass-to-charge ratio;

NIST: National Institute of Standards and Technology;

NTS: Nontranscribed sequence;

P-gp: P-glycoprotein;

PANSS: Positive and Negative Syndrome Scale;

PCA: Principal Component Analysis;

PCR: Polymerase chain reaction;

PET: Positron emission tomography;

PSI: Psychotomimetics States Inventory;

RFLP: Restriction fragment length polymorphism;

RNA: Ribonucleic acid;

RPM: Rotations per minute;

rRNA: Ribosomal ribonucleic acid;

SOFT-AAFS: Society of Forensic Toxicologists and The Toxicology Section of the American Academy of Forensic Sciences;

SPME: Solid-Phase Microextration;

SPSS: Statistical Package for Social Sciences;

SWGTOX: Scientific Working Group for Toxicology;

$t_{1/2}$: Half-Life;

TLC: Thin Layer Chromatography;

ToFMS: Time of Flight Mass Spectrometry;

UGT2B7: UDP-Glucuronosyltransferase-2B7;

USA: United States of America;

UV: Ultraviolet;

v/v: Volume concentration;

w/w: mass / mass (x100)

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Part I:

Introduction

1- *Salvia divinorum* and other natural products for recreational uses: brief overview

2- The plant, *Salvia divinorum*

3- The hallucinogenic compound, Salvinorin A

4- Potential therapeutic interest of salvinorin A derivatives

1- *Salvia divinorum* and other natural products for recreational uses: brief overview

Since synthetic drugs of abuse such as cocaine and amphetamine became scheduled under national and international drug laws, unregulated natural products have become more attractive for those who want to experience new psychotropic feelings and maximum enjoyment.

In this context, the opportunity to legally sell psychoactive drugs, gave the opportunity to retail stores, known as "smart shops" or "head shops", to increase their sales volume and capital gain. Also, the internet market for natural products intended to be used for recreational purposes has been an increasing reality (Arunotayanun & Gibbons, 2012).

One of the most popular natural legal drugs, in many countries, is *Salvia divinorum*. According to Schmidt et al. (2011), in websites headquartered in United Kingdom (country with the largest percentage of online suppliers in Europe), *Salvia* (*Salvia divinorum*) ranks the top 5 selling products alongside with Kratom (*Mitragyna speciosa*), Hawaiian Baby Woodrose seed (*Argyreia nervosa*), Fly agaric (*Amanita muscarita*) and Genie (chemically complex mixture of synthetic cannabinoids and plants).

Data from EMCDDA (2011a) revealed that European stores selling *Salvia divinorum* have raised from 72 to 110, from January 2011 to July 2011. This represents an increase of approximately 53%, in only 6 months. According to the same source, the average price also helps to develop customer loyalty: on average, 10 g of *Salvia divinorum* costs between 6-12€.

Besides the fact that natural products used as psychoactive drugs circumvent legal constraints, it is especially disturbing for scientific community the fact that these products do not provide information on eventual side effects, precautions, contraindications and most of the times, do not even provide reliable information on its qualitative and quantitative composition. This lack of information combined with the facility to buy these drugs and the similar psychoactive effects to those of synthetic ones, represents a threat, not only for users, but also for society in general.

2- The plant *Salvia divinorum*

2.1 Historical Background

The American continent is a geographic area known for its abundance in psychoactive mushrooms and plants. Actually, in America, it can be found many natural plants and mushrooms having hallucinogen properties, growing in forests, in fields, or in mountains. Archeological findings had proven the use of these mushrooms and plants over 5000 years in pre-Colombian period for magic and/or religious practices as well as for therapeutic purposes (Carod-Artal, 2011).

Hallucinogen plants used by Mesoamerican populations are commonly referred as entheogens, because of its mysticism stimulation and divine communication. The main goal of practices in which these plants are used, is to reach a state of trance. The altered state of consciousness level is characterized by time-space misdirection, inner peace sensation, hallucinations and a nature bond feeling. Mesoamerican religions recognize shaman as a person capable of maintain communication between physical and spiritual worlds, and the one who provide entheogens for religious ceremonies. The shaman uses several psychoactive substances for several purposes: contact with spirits, diagnose illnesses, insure good harvests or predict rain (Carod-Artal, 2011).

In the Mazatec indigenous culture of Oaxaca (Fig.1), the plants with spiritual connotation, and historically the most important, are *Salvia divinorum*, the *Teonanacatl* (also called "magic mushroom"), and hallucinogenic morning glory, also known as *Ololiuqui*. The name *"Salvia"* comes from the Latin word that means "to heal" (Imanshahidi & Hosseinzadeh, 2006). *Salvia divinorum* is a small perennial shrub specie of the mint family *Labiatae*, subgenus *Calosphace*, subgenera *Salvia*, native from the Northern Mexico, also known as "*Maria Pastora*", "The Diviner's Sage", "*Hierba Maria*" and "eye of the shepherdess", and has been used by Mazatec shamans in Oaxaca for over 3000 years (Turner, 2004).

Fig. 1 - The Sierra Mazateca located within southern Mexico. Adapted from http://neighborsabroad.org/wphome/?page_id=76

Salvinorin A (Fig.2) is the main psychoactive compound present in *Salvia divinorum*. It is a *kappa* opioid receptor (KOR) agonist (Imanshahidi & Hosseinzadeh, 2006). The lethargy induced by this plant was mainly used for divination practices. Nevertheless, shamans also use *Salvia divinorum* for therapeutic purposes, like headache, abdominal pain and diarrhea (Carod-Artal, 2011; Turner, 2004). Plant's leaves are usually ingested unchanged, or can be used to brew tea (Gonzalez et al., 2006; Halpern, 2004).

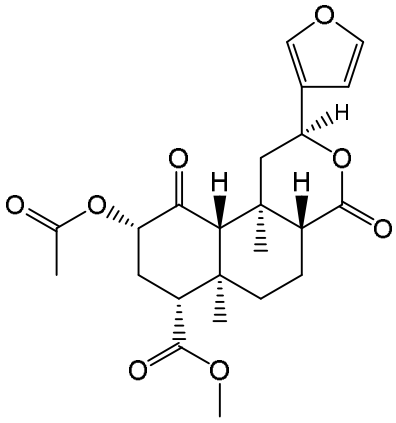


Fig. 2 - Salvinorin A Chemical Structure

Salvia divinorum was firstly described in western literature by the swedish anthropologist, Jean Basset Johnson, in 1939, who was particularly recognized by his previous studies on mushrooms used by earlier civilizations. *Salvia divinorum* was a very rare plant,

which was difficult to find in its natural ecosystem, the Sierra Mazateca mountains. However, due to its easy propagation by cuttings, *Salvia divinorum* has been cultivated in botanical gardens and private collections, in several countries. As *Cannabis*, *Salvia divinorum* (Fig.3) can be grown indoors, or in any humid and semitropical environment (Hoover et al., 2008; Turner, 2004).

Fig. 3 - Salvia divinorum plant. From http://psychotropicon.info/salvia-divinorum-diviners-sage-ska-maria-pastora/

The production of *Salvia divinorum* is inexpensive, being a great advantage for its intensive production. A small investment in fertilizers and solvents (for extraction of active compounds), combined with basic laboratory knowledge, makes the plant much more attractive to produce than its competitors, like Lysergic acid diethylamide (LSD) or phencyclidine derivatives (Valdes, 1994). It is believed that commercialized *Salvia divinorum* in circulation has been propagated from two parent clones of the species: one collected by R. Gordon Wasson in 1962, and the other collected by Bret Blosser, in 1991 (Turner, 2004). In fact, R. Gordon Wasson (ethnobotanist responsible for the revealing of the Mexican mushrooms to the modern world) and Albert Hofmann (renowned chemist involved in the discovery of LSD-25), were the first two persons to reap samples of *Salvia divinorum* and bring them to western civilizations, in 1962. In that year, both went to Oaxaca with the purpose of finding this mysterious plant used for religious and therapeutic purposes and carried the first specimens to United States of America (USA) (Halpern, 2004).

Mazatec Indians believe that *Salvia divinorum* is not an autoctone plant of this region. Actually, the few patches of known existence in Sierra Mazateca seem to be the result of

deliberate planting and anthropological distribution. It is possible that *Salvia divinorum* is a hybrid, however, there are no proven theories about its eventual prospective parents (Jenks. et al., 2011). It has been noticed that, among Mazatec Indians, the plant does not have an indigenous name. It is called "Diviner's Sage" or by other designations related to Christian customs like "the shepherdess" or "Mary". This may be explained by the birth or recognition of the plant, only after the decline of the Aztec Empire in the battles against the Spanish Army conducted by Hernán Cortés, in 1520. However, Gordon Wasson suggests another possibility: *Salvia divinorum* might also be the Aztec plant *Pipiltzintzintli*, an entheogen with very little available information, and supposed to be extinguished (Turner, 2004).

2.2 Phylogenetic Classification

Salvia divinorum has been phylogenetically classified in the family *Labiatae*, subgenus *Calosphace*, subgenera *Salvia*. Nevertheless, the phylogenetic classification of *Salvia divinorum* remains controversial.

There have been advances and retreats on the similarity of the specie *Salvia divinorum* to other plants classified in taxonomic sections *Dusenostachys* or *Tubiflorae*, even using different approaches, like molecular phylogenetics, floral morphology or any other (Epling & Játiva-M, 1962; Jenks, 2009) (Fig.4). Making use of recent technologies, studies involving DNA from chloroplasts and the entire nuclear ribosomal internal transcribed spacer region to determine phylogenetics tree, it was discarded the possibility to include *Salvia divinorum* in *Dusenostachys* section. In fact, in accordance with the mentioned tests, only *S. venulosa* species seems to be similar to *Salvia divinorum* among all the species of the *Calosphace* subgenus. *S. venulosa* is classified among other plants in the *Tubiflorae* section. Hence, according to Jenks. et al. (2011), *Salvia divinorum* should no longer be classified within *Dusenostachys*, and should be classified in a phylogenetic position nearest to *S. venulosa*.

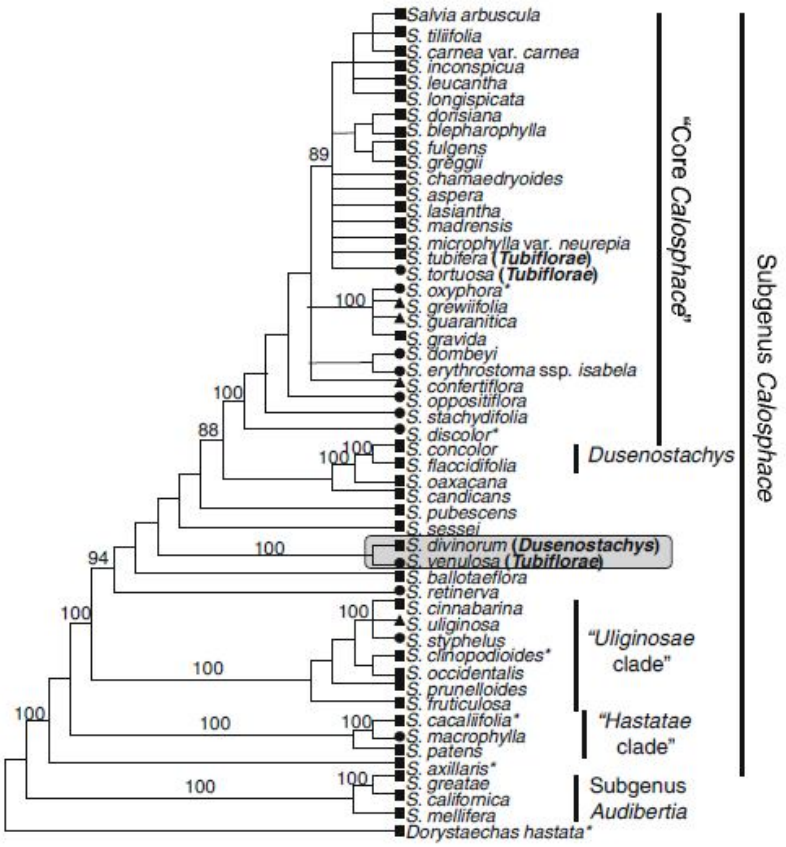


Fig. 4 - Phylogeny of Salvia divinorum. from Jenks A, Walker J, Kim S. Evolution and origins of the Mazatec hallucinogenic sage, Salvia divinorum (Lamiaceae): a molecular phylogenetic approach. Journal of Plant Research. 2011;124:593-600

2.3 Prevalence, Patterns of use and legal status

The prevalence of *Salvia divinorum* use at a global scale is, for the moment, unknown. Nevertheless, it seems particularly prevalent in Northern American countries, namely USA and Canada. Ford et al. (2011) estimated that 1.7% of adolescents from USA aged 12-17, used the substance until 2008–09, whereas, in Canada, the prevalence, in the same period, reached 6.2% (Currie, 2013).

Salvia divinorum was traditionally used by Mazatec Indians in Oaxaca for more than 3000 years for medical purposes (Valdes et al., 1983). However, in the late 1990s, the use of *Salvia divinorum* became more associated with recreational practices, rather than spiritual or therapeutic. Unlike those times, nowadays, the plant is not mainly chewed or ingested, but it is more often smoked in pipes, similarly to those commonly used with *Cannabis*. With this different administration route, the effects have also changed. When smoked, *Salvia divinorum* expresses its effects immediately and yields a dissociative sensation (Gonzalez et al., 2006; Stogner et al., 2012). Highly concentrated liquid extracts (like tinctures) are also available in different websites, as well as the leaves for chewing (Hoover et al., 2008).

Due to the worldwide globalized culture, the plant used in a restrict region of Oaxaca became quickly used at different places of the globe. Some studies have focused on the importance of the videos broadcasted in websites like YouTube™ , showing moments lived by anonymous people, after *Salvia divinorum* consumption (Halpern, 2004; Schmidt et al., 2011; Vohra et al., 2011). Also, in sites designed to divulgate and sell legal drugs, *Salvia divinorum* is one of the most widespread, followed by Kratom and some mushrooms (Gonzalez et al., 2006; Schmidt et al., 2011). Most *Salvia divinorum* consumers confirmed they first heard about the plant through friends, and only 40% admitted to have purchased the product themselves (Ford et al., 2011). *Salvia divinorum* is most commonly acquired in the Internet, in “smart shops” (also known as “head shops”), in music festivals or through drug dealers (Gonzalez et al., 2006; Schmidt et al., 2011; Vohra et al., 2011). Websites that promote *Salvia divinorum* usually attest the drug's safety, absence of adverse effects and mistaken effects of euphoria and analgesia (Hoover et al., 2008).

Ilgen et al. (2011) conducted a study in USA named "Monitoring the Future", that revealed the use of *Salvia divinorum* among teenager students, between 1987 and 2008. The study found out that 5.5% of the 12th graders, 3.7% of the 10th graders and 1.7% of the 8th graders, have already tried *Salvia divinorum*. In 2008, The National Survey on Drug Use and

Health claimed that 0.6% of the adolescents (aged 12-17) and 1.7% of young adults (aged 18-25) had used *Salvia divinorum* in the previous year (Ford et al., 2011). Among other similar studies, these results reveal the high prevalence among young adults. In summary, the prevalence studies that have been made reveal a high prevalence among young male adults, living on-campus, fraternity members, which are engaged in risk-taking behaviors (like selling illicit drugs and stealing), and that consume other drugs, especially *cannabis*. In fact, polydrug use is the most robust determinant of *S. divinorum* consumption. The percentages above cited indicate a similar and sometimes higher adhesion to *Salvia divinorum* than to LSD, ketamine, phencyclidine or dimethyltryptamine (Lange et al., 2008; Perron et al., 2012; Stogner et al., 2012; Wu et al., 2011).

Consumers of *Salvia divinorum,* using it as a recreational drug, face the same risks as users of other recreational drugs: possible future drug addiction, accidents caused by mental impairment, overdoses linked to inexperience drug intake and the long run effects of prolonged drug abuse (Pavarin, 2006).

Salvia divinorum became legally controlled, in 2003 by a municipal law in Saint Peters, Missouri, USA, that restricted *Salvia* sales. In August of 2005, Louisiana approved a law to implement the suppression of production, manufacture and distribution of *Salvia divinorum*, as well as other forty plants. Missouri, Maine, Tennessee, North Carolina, West Virginia, Wisconsin, Delaware, Oklahoma, North Dakota, Florida, Illinois, Kansas, Mississippi, Virginia, Hawaii, Nebraska, Ohio, South Dakota, Alabama, Georgia, Kentucky, Michigan, Minnesota, Connecticut, Indiana, Pennsylvania, Wyoming and Colorado are the other states that predict punishment for the use and/or production of *Salvia divinorum*, or its main compound salvinorin A (Stogner et al., 2012). The U.S. Drug Enforcement Administration has identified *Salvia divinorum* as a "drug of concern" since 2005, although, under U.S. federal law, it is not illegal to possess the drug. Countries such as Australia, Belgium, Denmark, Italy, Japan, Latvia, Lithuania, Romania, Sweden and some states of the US, included *Salvia divinorum* and salvinorin A in drugs legislation. Croatia, Germany, Poland and Spain only regulate the plant whereas in Estonia, Finland and Norway, *Salvia divinorum* is included in medicines legislation. In Canada it is legal to sell *Salvia divinorum* but only upon authorization under the Natural Health Products Regulation(EMCDDA, 2011b) (Fig.5).

Sumnall et al. (2011) performed a study in United Kingdom in which 154 recent users of *Salvia divinorum* were surveyed. About one quarter of participants reported that they started consuming *Salvia divinorum* as an alternative to illegal drugs. Nevertheless, the majority of the participants reported that they would continue to use the plant, even if it was illegal. The authors assumed that these data might be related to the fact that consumers attribute lower toxic effects to *Salvia divinorum* than to other similar drugs. It was concluded by these authors that legal controls would have no impact to dissuade former users to purchase the drug. Nevertheless, although the drug did not come to be eradicated, the fact of becoming socially less acceptable could have long term effects on consumption and sale.

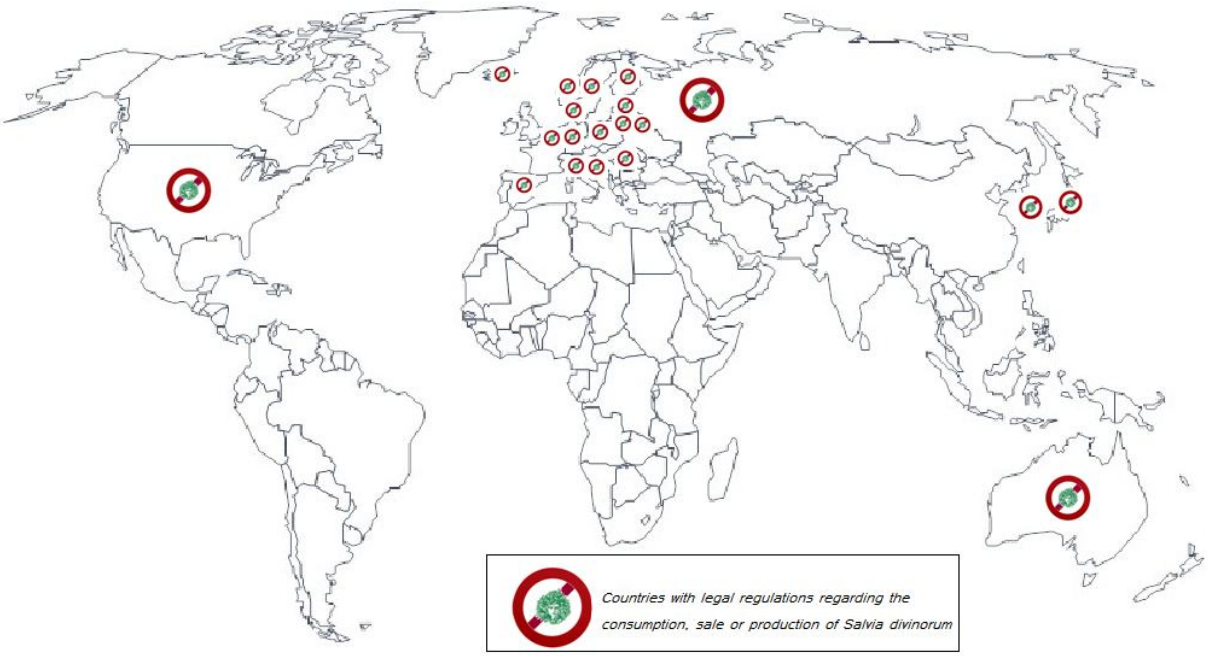


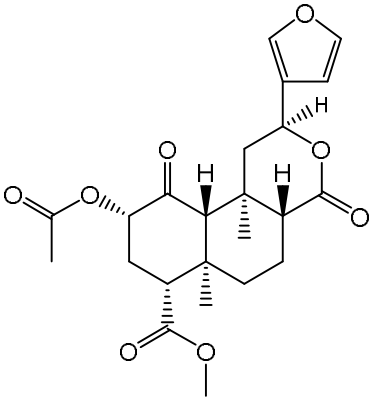
Fig. 5 - Countries with legal implications regarding Salvia divinorum until 2012

In Portugal, consumption and marketing Salvia *divinorum* had no restrictions, since its main compound (salvinorin A) was not included in national Decree Law 15/93 (DL15/93), that identifies legal status of trafficking and consumption of narcotic and psychotropic drugs (INFARMED, 1993). Nevertheless, in 17th April 2013, it was published the Decree Law 54/2013 that defined the prohibition over advertising, trading, production, importation, exportation, distribution, sale or possession of the new psychoactive substances. *Salvia divinorum* is one of the mentioned "new psychoactive substances" alongside with other plants such as *Mitragyna Speciosa*, *Amanita muscaria*, *Piper methysticum, Areca catechu* and Kava. Phenylethylamines, piperazines, cathinone derivatives, synthetic cannabinoids and analogs of cocaine were also targeted in the Decree Law 54/2013 (República, 2013).

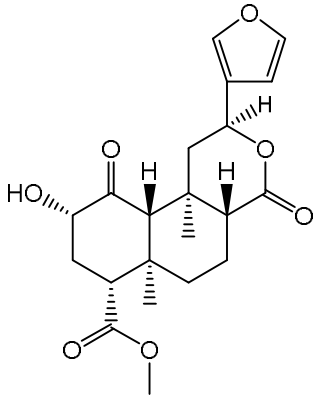
2.4 Other compounds identified in Salvia divinorum plant

Salvinorin A might be considered the most important active compound present in *Salvia divinorum*. Although this plant has other recognized chemical compounds, salvinorin A is present in much higher concentrations than the remaining compounds, which may be insignificant from the pharmacological point of view (Listos et al., 2011).

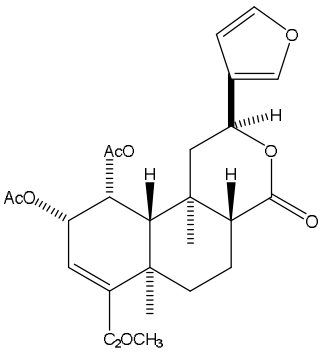
After the identification of salvinorin A and B, many other diterpenes were isolated from *Salvia divinorum* samples: salvinorin C (with weak affinity for KOR and none psychotropic effects in humans) (Valdes et al., 2001); salvinorins D-F (salvinorins D and E are believed to be precursors of salvinorin A) (Munro & Rizzacasa, 2003); salvinorin G (Lee et al., 2005); salvinorins H-I (Shirota et al., 2006); salvinorin J - biosynthetically, salvinorin J is probably derived from salvinorin I by an acetyltransferase action and might represent a key intermediate in a novel biosynthesis pathway of salvinorin A, via salvinorin I (Kutrzeba et al., 2010); divinatorins A-C (Bigham et al., 2003; Shirota et al., 2006); divinatorins D-E (Lee et al., 2005); salvinicin A and B (salvinicin B is mentioned as the first μ-opioid antagonist having a neo-clerodane skeleton) (Harding et al., 2005); and salvidivins A-D (Shirota et al., 2006) (Fig. 6). Among all chemical compounds mentioned, only salvinorin G, salvinicin A and divinatorin D had measurable affinities for KOR (Harding et al., 2005; Lee et al., 2005). Salvinorin A remains the only isolated neoclerodane diterpene linked to high affinity for KOR.



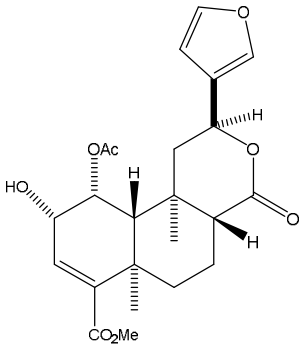
Salvinorin A



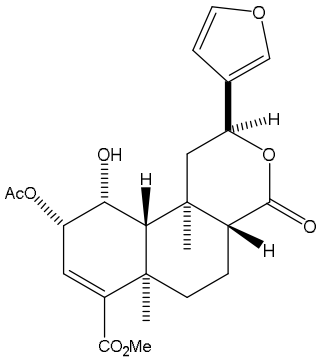
Salvinorin B



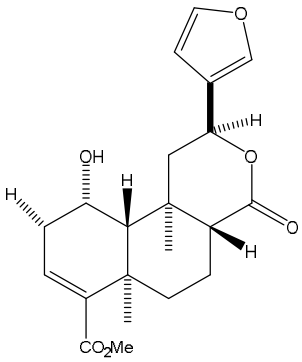
Salvinorin C



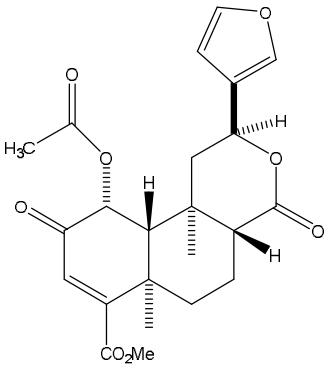
Salvinorin D



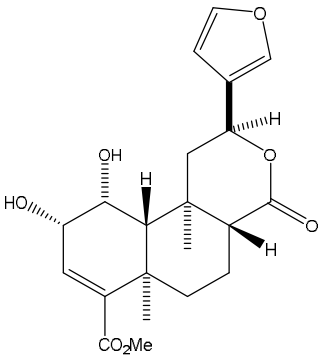
Salvinorin E



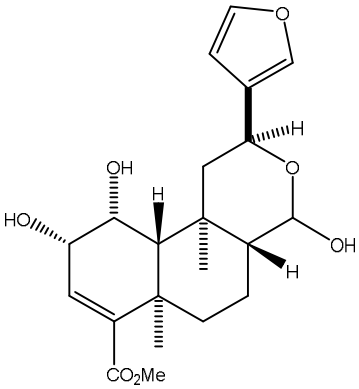
Salvinorin F



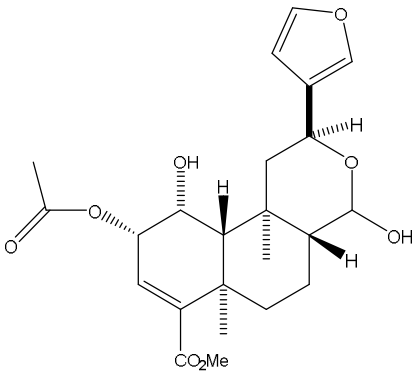
Salvinorin G



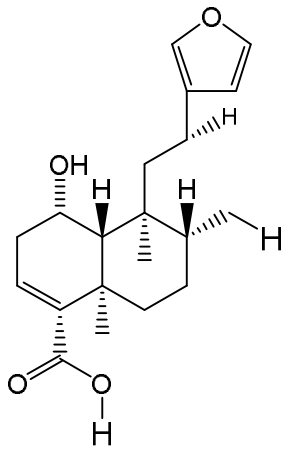
Salvinorin H



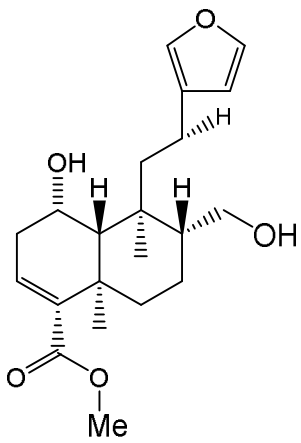
Salvinorin I



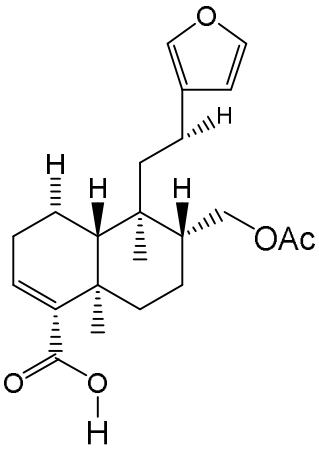
Salvinorin J



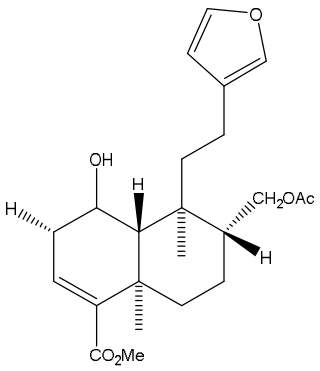
Divinatorin A



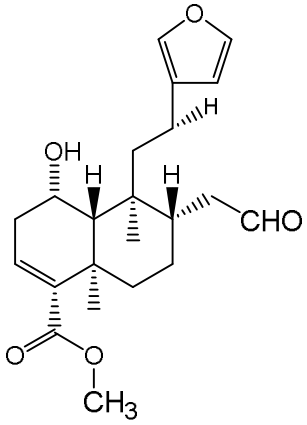
Divinatorin B



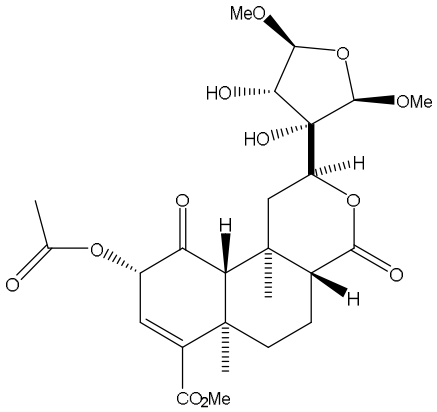
Divinatorin C



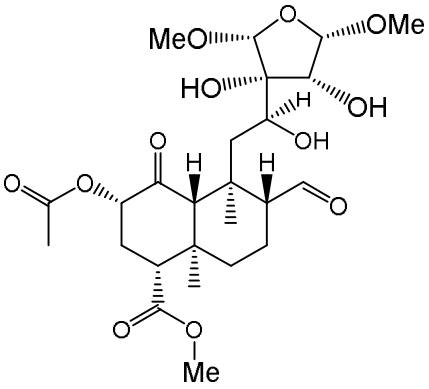
Divinatorin D



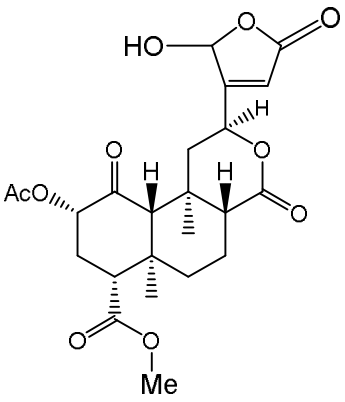
Divinatorin E



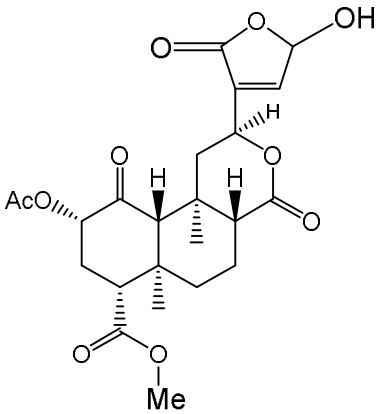
Salvinicin A



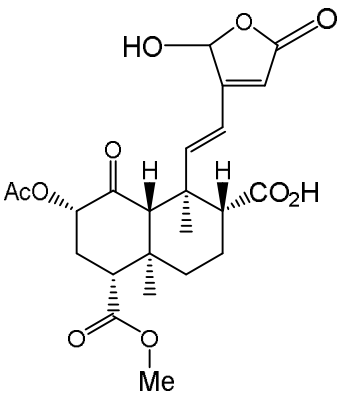
Salvinicin B



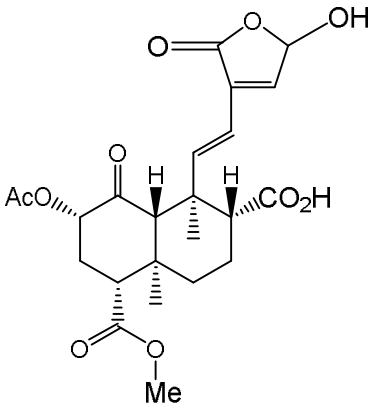
Salvidivin A



Salvidivin B



Salvidivin C



Salvidivin D

Fig. 6 - Chemical structures of the compounds already identified in *Salvia divinorum* plant

2.5 - Routes of Exposure

The traditional consumption of *Salvia divinorum* involved chewing the plant or brewing it as an infusion. Nowadays, it is used as a recreational drug, hence, the extract of the leaves is more often smoked (Siebert, 1994; Singh, 2007).

Siebert (1994) compared the effects of inhaled *Salvia divinorum* vapors, absorption of an alcohol-based spray of *Salvia divinorum* and the ingestion of capsules with the same quantity of plant. Inhalation of 200 μg as a vapor was the most efficient method. Through this administration route, the effects started to be expressed at the end of 30 seconds. The absorption of the spray, proved to be active, but only a small percentage of salvinorin A was absorbed. In this case, the compound also took about 30 seconds yielding psychoactivity. No psychoactivity was found following ingestion of the capsules with salvinorin A. The maximum dose administered by inhalation was 2600 μg. No acute or long-term negative effects were reported (Siebert, 1994). As later evidenced by Mendelson et al. (2011), the oral absorption of *Salvia divinorum* active compounds was very unlikely and irrelevant. No psychoactive effects were obtained in either studies of Siebert (1994) and Mendelson et al. (2011) by oral administration of *Salvia divinorum*, presumably due to the unreliable sublingual absorption of the plant's active compounds. In the study of Mendelson et al. (2011), performed with 8 adult subjects, 7 different doses were sublingual administered to each person, ranging from 0 to 4000 μg of salvinorin A, being the administrations separated by at least 24h. Even the higher concentration did not even produced mild effects. The different results obtained, collected through questionnaires between the experimental group (who had taken Salvinorin A) and the placebo group, were not statistically significant. When taken orally, salvinorin A seems to be degraded in the gastrointestinal tract and, as aforementioned, only a small dose of the drug suffers sublingual absorption. These data might explain the fact that, in traditional use, *Salvia divinorum* produces only mild effects whereas, through the most recent practices, the reported effects are much more intense (Imanshahidi & Hosseinzadeh, 2006).

A recent study performed by Addy (2012) confirmed the extension of self-reported effects in humans who had smoked *Salvia divinorum*. In this work, 32 volunteers smoked 25mg of plant material, using a smoking pipe. It was registered the blood pressure, heart rate, temperature and respiration rate. The effects produced by the drug were also evaluated through self-reported effects and observer-rated effects [1]. Observer-rated effects such as laugh and hyper movement were reported after the subjects smoke an active dose of

[1] Observer-rated behavior studies – Studies in which the subjects behavior is evaluated and classified, by a specialist, after some kind of stimulation (chemical, physical, environmental, or any other).

salvinorin A, however, no changes in blood pressure, heart rate, temperature or respiration rate occurred. Even knowing that a small dose of smoked salvinorin A is enough to produce several of the drug's effects, it should be taken into account that there are variations in smoking techniques and the instruments used may interfere with combustion of the drug, varying the dose administered. Therefore, the attribution of a minimum dose value to elicit psychoactivity, should be made carefully (Mendelson et al., 2011).

2.6 Salvia divinorum psychoactive effects

2.6.1 – Effects on animal models

Some experiments, namely the forced swimming test[2] with rodents (rats and mice), revealed decrease of mobility after administration of *Salvia divinorum*, thus contradicting the effects reported by Lange et al. (2010) in humans, mentioned earlier (John et al., 2006). These results might be related to the fact that high doses were used in these cases. In fact, small doses seem to promote the hyper-movement (acting as stimulants and increasing dopamine levels), while high doses seem to promote hypo-movement (acting as depressing and diminishing dopamine levels) (Baker et al., 2009; Braida et al., 2007; Carlezon et al., 2006).

In contrast to other drugs, *Salvia divinorum* has a low addictive liability, being unlikely to be used compulsively, repetitively, or persistently. This might be related to the fact that addictive drugs usually increase dopamine in the *nucleus accumbens*, contrary to what seems to happen with *Salvia divinorum*. According to studies in rodents, *Salvia divinorum* decreases dopamine levels (likely to what has been demonstrated with other KOR agonists) (John et al., 2006; Willmore-Fordham et al., 2007; Zhang et al., 2005). These results encourage the possible benefit of use *Salvia divinorum* to treat drug dependence (for instance, with cocaine), mainly due to the effects of salvinorin A on dopamine-mediated receptors (Johnson et al., 2011; Morani et al., 2009).

In studies performed with mice, salvinorin A caused antinociception in tail flick[3], hot plate[4] and acetic acid[5], reinforcing the potential analgesic effect of *Salvia*'s most active compound (Ansonoff et al., 2006; John et al., 2006).

2.6.2 – Effects reported in humans

Most information concerning psychoactive effects of *Salvia divinorum* are obtained from questionnaires and the answers frequently extol the "intense" and "unique" effects of the drug (Lange et al., 2010; MacLean et al., 2013) ranging the required dose to produce hallucinogenic effects from 200 μg to 600 μg of salvinorin A (Siebert, 1994).

[2] Forced Swimming Test –In this test the animal is submitted to trials in which he tries to escape from an acrylic glass cylinder filled with water. The time that the animal spends without moving is supposed to be decreased by antidepressants.
[3] Tail Flick Test – Evaluation of the nociceptive response latency in rats, with recording of time between the time when a light beam is focused on its still tail, and the time that it begins to move.
[4] Hot Plate Test - Evaluation of the nociceptive response latency to hind paw licking in mice, when these are placed on a heated metal plate.
[5] The acetic acid induced writhing – Evaluation of the response to mediate peripheral pain, induced by peritoneal injection of acetic acid, from abdominal contractions in mice.

The fast onset of *Salvia divinorum*'s effects is remarkable, being noted in seconds or few minutes (Baggott et al., 2010; Ranganathan et al., 2012).

Gonzalez et al. (2006) reported a questionnaire about the psychoactive experience lived during the consumption of this drug, translated into 75% of the responses between "intense" to "very intense" and "very intense" to "extremely intense". The answer "moderate" collected 19% of the responses, while the word "light" was chosen only in 6% of cases. The positive effects more commonly cited were the trip (41%), followed by euphoria (28%) and dissociative effects (19%). The negative effect more frequently mentioned was the short duration (38%). The results reflect the main goals of this drug consumption as well as the expectations of its consumers.

Salvia divinorum induces significant alterations in behavior and cognition. Some effects of *Salvia divinorum* are consistent, in different studies, such as "hyper-movement", "emotional effects" (especially fear), "speech effects" and "heating effects" (the users revealed feeling of increasing temperature during the experiment) (Addy, 2012; Johnson et al., 2011). However, although *Salvia* users' respondents assumed to feel high temperature during the experiment, recorded rectal temperature decreased in some studies (Ansonoff et al., 2006).

Short-lasting depersonalization, visual and hearing hallucinogenic effects (sense of becoming objects, visions of two-dimensional surfaces, motion sensations and overlapping realities, perceptual distortions and profoundly altered sense of self and environment) are quite marked, and users classify them as unique and substantially different from other hallucinogens (Baggott et al., 2010; MacLean et al., 2013). The dissociative effects are evident for moderate and high doses (MacLean et al., 2013; Ranganathan et al., 2012). Some other described effects of *Salvia divinorum* consisting on hysterical laughter and feelings of transformation into people or animal, being in multiple places at the same time and even levitation (Singh, 2007).

It was found an inverse correlation between the dose and recognition accuracy. The follow up after the screening has not shown any signs of depressive effects, anxiety, confusion, psychiatric symptoms or visual disturbances (MacLean et al., 2013). These results corroborate those obtained by Gonzalez et al. (2006), where users usually claim positive after-effects, such as increased insight and improved mood. A study performed by Baggott et al. (2010) revealed that more than 24h after *Salvia divinorum* consumption, 25.8% of the participants reported positive effects and 46.5% reported improved mood. Regarding to the most persisting effects

over a shorter time frame, 44.8% of the individuals reported improved mood and 42.6% reported a feeling of calmness.

In another study, in which individuals were monitored, no serious adverse effects (death, hospital stay or emergency room visit) occurred; being drowsiness and dizziness the only adverse effects registered (Baggott et al., 2010). The first reported case of a persistent negative outcome related to the use of *Salvia divinorum* was related in 2009. A 21 years old person evidenced persistent psychosis and paranoia after smoking *Salvia divinorum*. Nevertheless, the medical team suspected that the patient was genetically predisposed to schizophrenia, and *Salvia* might have precipitated the clinical manifestations (Przekop & Lee, 2009). The "loss of awareness" that might result in users hurting themselves or others (Killinger et al., 2010), is another consequence linked to *Salvia divinorum* consumption. Although, there have been only few documented negative occurrences associated with *Salvia* use and the eventual long-term dependence seem to be unlikely, the coordination loss, failure on speech, interpersonal impairments and loss of self-care should be valued as an increased risk to users and those around them (risks of injury, interpersonal conflict and property damage)(Lange et al., 2010).

Nevertheless, there is little data on human experimentation with *Salvia divinorum*, and works presented in literature indicate different routes of administration, different doses, and lack characterization of the subject samples (Ranganathan et al., 2012).

3- The hallucinogenic compound Salvinorin A

3.1. Physicochemical properties

Salvinorin A was isolated for the first time by Ortega et al. (1982), and chemically characterized by nuclear magnetic resonance and single-crystal X-ray analysis. It was considered to be psychoactive for the first time in the 1990s (Valdes, 1994). The relationship between salvinorin A and the *kappa* opiate receptor was firstly established in 2002 (Roth et al., 2002).

Salvinorin A remains the most potent naturally occurring hallucinogen, being chemically and structurally unique: it is the first known psychoactive diterpene and the first non-nitrogenous hallucinogen (Killinger et al., 2010).

Salvinorin A is chemically named as (2S,4aR,6aR,7R,9S,10aS,10bR)-9-(acetyloxy)-2-(3-furanyl)dodechydro-6a,10b-dimethyl-4,10-dioxo-2H-naphtho[2,1-c]pyran-7-carboxylic acid methyl ester, and has the empirical formula $C_{23}H_{28}O_8$ (Fig. 7).

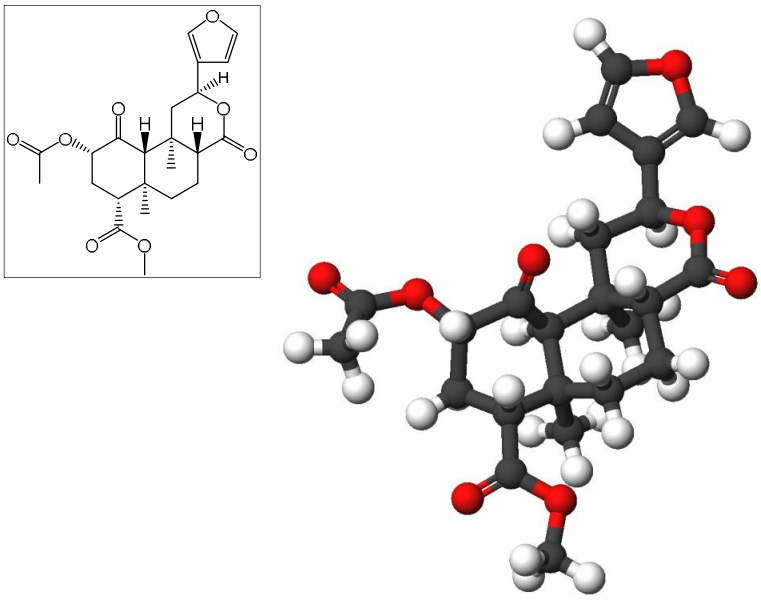


Fig. 7 - Salvinorin A 3D Chemical Structure. From http://commons.wikimedia.org/wiki/File:Salvinorin_A-sticks.png

The molecular weight of salvinorin A is 432.46 g/mol. Regarding physical data, the melting point of salvinorin A lies between 238-244 °C and the boiling point is 760.2 °C. Salvinorin A is thermo unstable, so it must be stored at -20 °C.

Salvinorin A is unstable in basic solutions and has a high solubility in organic solvents, such as acetone, chloroform, acetonitrile and methanol. It is however insoluble in hexane and water.

Many attempts have been made to characterize *Salvia divinorum* through the detection of its most important compound, Salvinorin A. The UV spectrum of a methanolic solution of salvinorin A exhibits a peak at 211 nm. The characteristic *m/z* ions of salvinorin A are *m/z* 94, *m/z* 55, *m/z* 121, *m/z* 107, *m/z* 273, *m/z* 166, *m/z* 220, *m/z* 252, *m/z* 234, *m/z* 359, *m/z* 318, *m/z* 404 and *m/z* 432 (in decreasing abundance) (EMCDDA, 2011b; Sigma-Aldrich, 2011).

3.2 Pharmacokinetics and pharmacodynamics of salvinorin A

In vivo data, using humans and animal models, and *in vitro* methods have been crucial for the determination of the pharmacokinetics and pharmacodynamics of *Salvia divinorum* and its major constituent salvinorin A, as well as to elucidate its pharmacologic and toxicological effects. Nevertheless, these works are yet very scarce and further information is needed to elucidate the biological mechanisms involved.

3.2.1.1 – Studies in animal models

Teksin et al. (2009) demonstrated that, after administration, salvinorin A is absorbed in the lungs, reaches the systemic circulation, crosses the blood brain barrier and accumulates in the central nervous system. The pharmacokinetic study was performed in male Sprague-Dawley rats. The animals were submitted to a single administration of 10mg/kg, and after euthanized, blood samples were collected and brain tissue was analyzed. Salvinorin A can easily cross the blood barrier due to its low molecular weight (432.36 g/mol) and high lipophilicity.

Positron emission tomography (PET) studies developed in baboons by labeling salvinorin A with carbon-11 revealed that salvinorin A easily crosses the brain blood barrier, taking only 40 seconds to reach 3.3% of the injected dose. After the baboons were anesthetized with intramuscular ketamine hydrochloride, a dose of 250mg of salvinorin A was administered through a catheter placed in a radial arm vein. It was demonstrated that the compound was distributed throughout the brain, with a high concentration in the cerebellum and cortex. High concentration of the hallucinogenic drug in cerebellum and visual cortex, might justify the behavioral effects portrayed when the drug is inhaled. The referred PET studies also estimated that less than 10 μg of salvinorin A in the human brain could be enough to promote its psychoactive effects, since in previous studies of Siebert (1994) it was shown that 200 μg of smoked salvinorin A was enough to promote the referred effects, and the maximum average brain concentration of salvinorin A corresponded to 3,3% of the administered dose (Hooker et al., 2008).

3.2.1.2 – Studies in humans

In a study performed by Gonzalez et al. (2006) with 32 recreational users of *Salvia divinorum*, 31% of the surveyed, have considered the onset of effects "instantaneous" and, 57% considered it "less than a minute". These answers were obtained through a retrospective assessment about the most recent *Salvia divinorum* consumption, and all the subjects revealed to have experienced psychotropic effects.

3.2.2 – Metabolism and Excretion

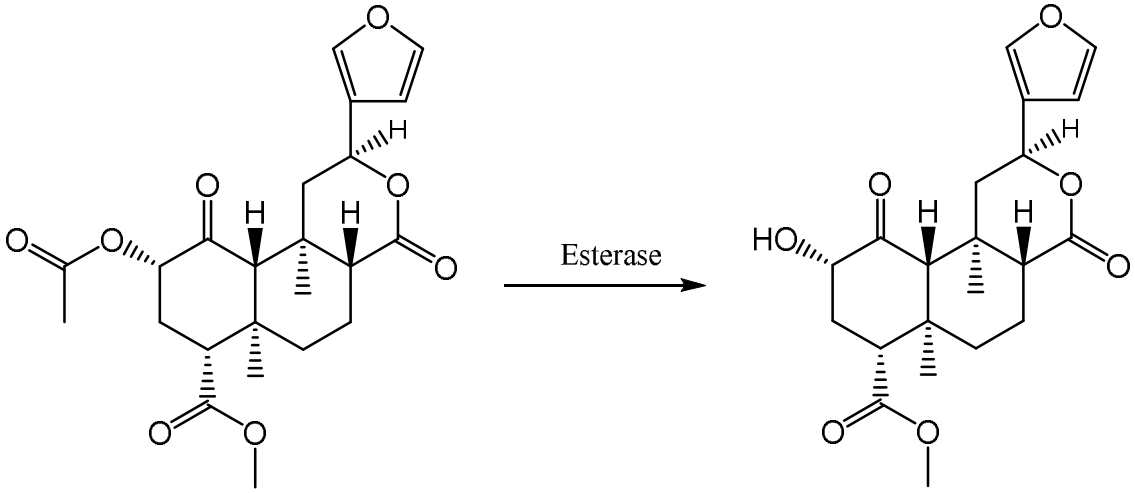


Fig. 8 - Hydrolysis of Salvinorin A into Salvinorin B

3.2.2.1 – Studies *in vitro*

Tsujikawa et al. (2009), have shown that 24h after administration in rats plasma, salvinorin A concentration decreased while salvinorin B concentration increased. Fresh whole blood was collected from anaesthetized male Wistar rats, plasma was separated by centrifugation and it was added 20µl of a salvinorin A solution. It is important to refer that the sum of the residual percentages of salvinorin A and salvinorin B did not attain 100%. These findings suggested that subsequent salvinorin B degradation occurred and/or there were other metabolic pathways of salvinorin A. Further analyses enabled to recognize a lactone-ring open

form of salvinorin B as its presumptive metabolite. In the same work, and in order to discover which esterase was responsible for salvinorin A degradation, various esterase inhibitors were experimented. The data obtained suggested that carboxylesterase would be the main responsible for that transformation since bis-p-nitrophenylphosphate (a carboxylesterase inhibitor) inhibited its hydrolytic activity (Fig. 8).

Studies developed by Schmidt et al. (2005) in fresh whole blood taken from adults rhesus monkeys, showed that salvinorin B is the most representative metabolite of salvinorin A, resulting from the ester hydrolysis at the 2-acetoxy group by an esterase in blood. It was found an inversely proportional relationship between the concentrations for salvinorin A and salvinorin B (Fig.8).

Teksin et al. (2009) demonstrated that salvinorin A is a substrate to the glycoprotein P. To enable the *in vitro* study of the glycoprotein P influence on salvinorin A efflux, MDCK-MDR1 cell line was used as model since it encodes high levels of glycoprotein P. High secretory transport of salvinorin A of $4.07 \pm 1.34 \times 10^{-5}$ cm/s was observed. It is, however, important to relate these results with the behavior *in vivo*. Salvinorin A has, as already mentioned, high lipophilicity, potency and easy permeability over barriers, which probably outweigh the effect of P-gp-mediated efflux on the central nervous system levels.

In vitro concentrations of salvinorin A significantly decreased after being exposed to the presence of the enzymes CYP2D6, CYP1A1, CYP2C18 and CYP2E1. These findings support the fact that salvinorin A is a substrate of CYP450. Glucuronosyltransferases enzymes, like UGT2B7, also seemed to diminish salvinorin A's concentration, which might be associated to the ester group of salvinorin A, a potential site for glucuronidation, by those enzymes (Teksin et al., 2009).

3.2.2.2 – Studies *in vivo*

Salvinorin A, in rhesus monkeys studies, has been estimated to have an elimination t ½ of 56.6 ± 24.8 min, after intravenous administration (Schmidt et al., 2005). This fast elimination time, suggests a likely hysteresis and a consequent single-dose tolerance to the drug. Other studies performed with baboons showed an even faster elimination: half-time estimated of 8 min, also after intravenous administration (Hooker et al., 2008). If the drug is so quickly eliminated, it is easy to understand why smoking is the preferred route of administration

nowadays: fast absorption and easy re-administration. This profile corresponds to the expectations of most of *Salvia divinorum* consumers (Baggott et al., 2010).

It was tested the metabolism comparison among salvinorin A, salvinorin B, and salvinorin B ethoxymethyl ether (EOM-SB), a derivative with greater potency and resistance to metabolism in baboon brain. In order to perform this study, two male Sprague-Dawley rats were anaesthetized and injected with a solution with salvinorin A in the peritoneum. After that, PET imaging was performed. It would be expected that EOM-SB exhibit slow uptake and clearance in brain, since it was seen as the most resistant to metabolism, especially when compared with salvinorin A. Nevertheless, the three compounds revealed very similar pharmacokinetics in brain, concerning uptake and clearance. The obtained results showed that metabolism is not the only responsible for the brief brain residence of salvinorin A. On the other hand, whole-brain EOM-SB concentrations diminished more slowly than the concentration of the other two compounds after intraperitoneal administration (Hooker et al., 2009).

3.3 Physiological and toxicological properties of salvinorin A

Despite the fact that all plants of the genus *Salvia ssp.* possess a wide range of pharmacological activities, such as sedative, hypnotic, muscle relaxant, analgesic, anticonvulsant and neuroprotective, *Salvia divinorum* has been the only species that exert hallucinogenetic activities, since it is the only one containing salvinorin A (Grundmann et al., 2007). Salvinorin A has a psychotropic activity in humans at low doses ranging between 200 and 500 μg, when vaporized and inhaled, making it the most potent compound occurring in a hallucinogenic plant (Siebert, 1994).

Salvinorin A is a neoclerodane diterpenoid which acts as a *kappa* opioid receptor agonist (Lovell et al., 2012; Roth et al., 2002). This fact made clear that its mechanism of action is different from the other hallucinogenic drugs. It was proven that salvinorin A is efficient in particular transduction systems and has a reduced propensity to cause receptor desensitization (Roth et al., 2002).

It has also been restated the absence of a basic nitrogen in the chemical structure of salvinorin A (Fig. 8), believed to be crucial in traditional opioid pharmacology, as reported for morphine, codeine and heroine molecules (Fig.9) (Lovell et al., 2012; Vortherms & Roth, 2006). Therefore, salvinorin A represents the first non-alkaloid opioid receptor type-selective drug (Lee et al., 2010).

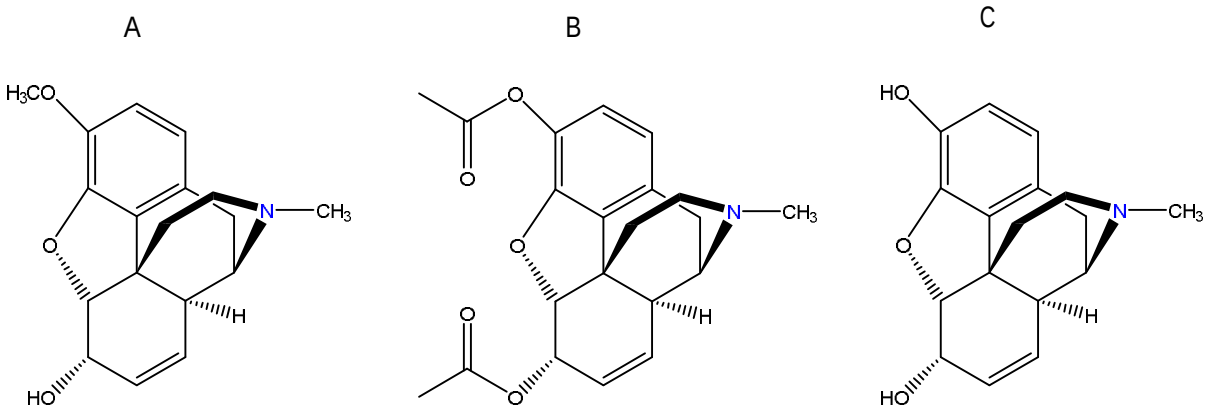


Fig. 9 - Codein (A) , heroin (B) and morphine (C) chemical structure evidencing the presence of Nitrogen (blue colored)

Salvinorin A has a high selectivity for *kappa* opioid receptors, having no activity in most of other systems, such as serotonergic, or in N-Metil-D-Aspartate receptors (Butelman et al.,

2004; Butelman et al., 2007). Data about structure/activity relationship, suggested that it is the carbon in the 2-position the main critical site for *kappa* opioid receptor binding and activation (Prisinzano, 2005).

3.3.1 – Studies in cell lines

Chavkin et al. (2004) compared the agonist activity of *Salvia divinorum* with dynorphin A, an endogenous neurotransmitter of the *kappa* opioid receptor, by measuring potassium conductance through G protein-gated K^+ channels in human embryonic kidney cells and oocytes. The results obtained suggest that salvinorin A was equieffective and equipotent to dynorphin A. In the same research, salvinorin A expressed itself as more potent than dynorphin A and have higher efficacy than U50.488 and U69.593.

Some *in vitro* studies have shown that salvinorin A does not interact with endocannabinoid system. Salvinorin A has no influence on calcium ion flux in hCB_1 receptors, and did not displaced a radiolabeled CB_1 receptor agonist ($[^3H]$-CP55,940) or a CB_1 receptor antagonist ($[^3H]$-SR141716), from hCB_1 receptors expressed in chinese hamster ovary cells. In this work it was hypothesized that salvinorin A does not act directly on cannabinoid receptors, but could act by indirect pathways (Walentiny et al., 2010).

3.3.2 – Studies in animal models

Roth et al. (2002) demonstrated, by the first time, the interaction between salvinorin A and *kappa* opioid receptors. These authors measured the inhibition of forskolin-stimulated cyclic adenosine monophosphate (cAMP) in presence of salvinorin A and the intracellular Ca^{2+} mobilization in *kappa* opioid receptors expressed in guinea pig brain.

Other studies also suggested that the mechanisms underlying salvinorin A psychoactive effects are quite different from serotonergic or glutamatergic hallucinogens, since salvinorin A failed to substitute other drugs like LSD or ketamine in Sprague-Dawley rats (Killinger et al., 2010). Nevertheless, the range of effects produced by salvinorin A has been considered similar to those produced by LSD, the main difference being the extremely short duration (Gonzalez et al., 2006; Listos et al., 2011). Corroborating those findings, other studies performed with *rhesus monkeys*, also showed that salvinorin A was antagonized by nalmefene (a μ and *kappa* receptor antagonist), since sedation and postural effects, evoked by salvinorin

A administration, were prevented by nalmefene IV injection (0.1 mg/Kg). However, ketanserin, a selective 5-HT_2 antagonist, failed to prevent the same sedation and postural effects. Classical psychedelics, like the already mentioned LSD, exert effects on the 5-HT_{2A} serotonin receptor (Butelman et al., 2009).

The evidence of salvinorin A being a *kappa* receptor agonist has been reinforced in studies with several animal models (like zebrafish, *rhesus monkeys* and rodents) trained to discriminate potent *kappa* agonists, such as U50.488 and U69.593 (Baker et al., 2009; Willmore-Fordham et al., 2007). Moreover, the effects produced by salvinorin A became attenuated after administration of nor-binaltorphimine (nor-BNI) and quadazocine, *kappa* opioid antagonists, to rats (Willmore-Fordham et al., 2007) and rhesus monkeys (Butelman et al., 2004), respectively. The stimulation of *kappa* opioid receptors in brain (especially in cerebellum) and spinal cord by salvinorin A, also justifies its ability to induce antinociception in mice, measured by using the tail-flick test (Grundmann et al., 2007; John et al., 2006).

While most *kappa* opioid agonists produce conditioned place aversion and decrease in locomotor activity, experiments with salvinorin A revealed an induction of conditioned place preference[6] and increase the spontaneous locomotor activity[7] of rodents, which allow to underline the possibility of other mechanisms be involved in the pharmacological effects of salvinorin A (Listos et al., 2011). It is hypothesized a new mechanism of action of binding and activation, not involving ionic interactions but hydrophobic interactions. The lack of strong ionic links and the existence of multiplicity of lipophilic binding sites, in the *kappa* opioid receptors, suggest that salvinorin A probably does not follow only one model (Grundmann et al., 2007).

Salvinorin A has shown to modify dopaminergic pathways, namely decreasing dopamine levels in the *caudate putamen* and decreasing dopamine neurotransmission levels in the *dorsal striatum*, mostly by affecting dopamine release but not dopamine uptake. The intrastriatal administration of salvinorin A results in decreased dopamine levels in rats *dorsal striatum*, which is due to the activation of *kappa* opioid receptors, since pretreatment with selective *kappa* opioid antagonists, like nor-BNI, attenuated the evoked decrease in dopamine overflow (Braida et al., 2008; Gehrke et al., 2008; Listos et al., 2011). Similar results were obtained by Gehrke et al. (2008).

It has also been proven that salvinorin A has affinity for dopamine D_2 receptors, since its intraperitoneal administration in mice, decreased dopamine levels in caudate putamen. Nor-BNI blocked the effect of salvinorin A on dopamine levels of mice, corroborating the idea

[6] Conditioned Place Preference Test – Condition used to study the rewarding and aversive effects of drugs. The animal might choose to move to one of two different apparatus, having in one of them the administered stimuli, and in the other the vehicle. The time the animal spends in any of the apparatus is measured to evaluate if a conditioned place preference was found.

[7] Open Field Test - Qualitative and quantitative measure of general locomotor activity and willingness to explore in rodents, after a drug administration.

of affinity of salvinorin A with dopamine D_2 receptors (Willmore-Fordham et al., 2007). Nevertheless, although high doses of salvinorin A produced a decrease in dopamine levels, low doses of salvinorin A seemed to produce an increase in dopamine levels. Unfortunately, the full mechanism of connections between k-opioid receptors, dopamine structures and salvinorin A are not totally understood (Braida et al., 2008; Listos et al., 2011).

Some studies tested the hypothetical interaction between salvinorin A and the endocannabinoid system (Braida et al., 2008; Butelman et al., 2009). Rimonabant (a cannabinoid receptor antagonist) reverses rewarding effects of a low dose administration of salvinorin A, evidenced in place preference test, with rats and mice (Braida et al., 2009). However, *in vivo* studies revealed significantly different behavior patterns after administration of salvinorin A and cannabinoids, such as tetrahydrocannabinol. Mice treated with salvinorin A had less hypothermia and catalepsya than those treated with tetrahydrocannabinol. Salvinorin A also failed to substitute tetrahydrocannabinol, in mice (Walentiny et al., 2010).

First studies about salvinorin A addictive properties concluded that it did not induce dependence. Nevertheless, behavioral studies in Wistar rats evidenced addictive effects. The rewarding effect of the compound was found after administration of low doses (0.1, 10 and 40 μg/kg) (Braida et al., 2008).

In opposite to other psychoactive drugs, such as synthetic amphetamine derivatives, which have critical severe adverse toxic effects, the consumption of salvinorin A does not appear to have relevant physiologic side effects. Mowry et al. (2003), in a study with Swiss-Webster mice showed minimal changes in body temperature, sympathetic nervous system activity and worthless cardiac consequences, after administering 1600 μg/Kg of salvinorin A. Regarding histological modifications, no changes were observed in tissues from liver, spleen, kidney, bone marrow or brain.

Notwithstanding, there are not currently many studies concerning toxic effects of salvinorin A. Some data predicted that it might be a reproductive toxicant in mammals (rabbits, rats and mice), but the information in this matter is scarce (Simpson et al., 2009).

3.3.3 – Studies in humans

The evaluation of endocrine effects, after salvinorin A inhalation, in a cohort of 10 individuals, revealed high levels of prolactin (probably because salvinorin A lowers dopamine via KOR agonism) and cortisol in plasma. These results demonstrate the stimulation induced by

salvinorin A to the hypothalamic-pituitary-axis activity. Psychoactive effects, measured by the Positive and Negative Syndrome Scale (PANSS) and Psychotomimetics States Inventory (PSI), were very clear and, in accordance with effects of δ-9-tetrahydrocannabinol and ketamine (Ranganathan et al., 2012).

In a survey with eight individuals (adults), the participants inhaled salvinorin A in crescent doses of 0.375-21 μg/Kg no changes in blood pressure, heart rate and tremors were observed. Anxiety rate during monitoring in human surveys was also low (MacLean et al., 2013).

In a recent case report, a 51 years woman who smoked 3-5 cigarettes of *Salvia divinorum* for 3-4 months, experienced vomits, nausea, diarrhea and abdominal discomfort, 48h after quitting the drug. This case evidenced possible withdrawal syndromes of chronic use of *Salvia divinorum* which might became more and more frequent with the widespread use of this plant (Travis et al., 2012).

3.4 Therapeutic effects of salvinorin A

3.4.1 – *Ex vivo* studies

Aviello et al. (2011), performed some studies using a cell line of peritoneal macrophages collected from mice, showing that salvinorin A has an ultrapotent effect on inflammatory response, experimentally caused by intraperitoneal injection with 10% sterile thioglycollate medium. These findings might be useful to diminish acute inflammation, and were observed after lipopolysaccharide paw edema and inflammation-sustained pain, induced in imprinting control region. Usually, during an inflammatory response, nitric oxide levels are high, however after administration of salvinorin A, the levels of nitric oxide and relevant metabolites were much lower. In order to understand the extension of effects of salvinorin A on inflammatory response, it was compared with the effectiveness of dexamethasone. Salvinorin A proved to be more effective.

3.4.2 – Studies in animal models

Salvia divinorum was used by the Mazatec Indians for healing some gastrointestinal problems. It is attributed to *Salvia divinorum* infusions, curative properties in a disease called "panzon de barrego" (swollen abdomen) as well as for relieving diarrhea (Capasso et al., 2008; Fichna et al., 2009). It has also been attributed to salvinorin A the inhibition of enteric cholinergic excitatory transmission, thus inhibiting contractility in the isolated guinea pig ileum. In the presence of salvinorin A, the twitch response induced by electrical field stimulation, mediated by acetylcholine from myenteric nerves, remained unchanged (Capasso et al., 2006). Some studies, performed in mice, showed that salvinorin A reduced the intestinal transit, through inhibition of ilea smooth muscle hypercontractility, by binding to KOR. Salvinorin A inhibits colonic motility, through the inhibition of neurogenic active ion transport, in mouse colon (Fichna et al., 2011; Fichna et al., 2009).

These findings might be relevant for the hypothetical importance of salvinorin A in the treatment of hypermotility of gastrointestinal motor function during endotoxemia, which occurs during gram-negative bacteria infections. Salvinorin A has the further advantage of preventing epithelial barrier dysfunction, as smooth muscle contractions of the colon decrease (Fichna et al., 2011; Fichna et al., 2009).

In a study in which chronic pain was induced in mice by formalin injection, salvinorin A has demonstrated to reduce mechanical allodynic effect and has elicited a reduction of spinal neuron hiperexcitability associated with chronic pain development. Salvinorin A has a positive effect in the treatment of inflammatory process and edema (Guida et al., 2012). However, it is important to recall that the potent analgesic effects of the *kappa* opioid receptors agonists is associated with many negative side effects like diuresis, sedation and psycothomimesis (John et al., 2006).

Salvinorin A anxiolytic/antidepressant potential has been studied in rodents. It was demonstrated that salvinorin A reduced anxiety (shown in the elevated plus-maze test [8]) and depression (measured by forced swimming test, locomotor activity and tail suspension test[9]) by its interaction with *kappa* opioid receptors and endocannabinoid system (Braida et al., 2009). More studies are needed to understand these biochemical interactions, since antidepressant effects may not be always the result of salvinorin A administration. In fact, few studies displayed depressive-like effects of salvinorin A depending on the administered dose (Braida et al., 2009; Carlezon et al., 2006).

[8] Plus-Maze Test – This test uses a plus-shaped apparatus with two open and two enclosed arms, each with an open roof, and is based on the rodent's aversion of open spaces. The raise of anxiety leads the mice to confining its movements to the enclosed spaces.
[9] Tail Suspension Test – In this test, the rodent is suspended by the tail from a lever and the movements of the animal are recorded. It occurs immobility moments and agitation periods. Usually, antidepressants induce decreased duration of immobility.

3.5- Potential therapeutic interest of salvinorin A derivatives

Towards obtaining better clinical results, many modifications and substitutions in the primary chemical structure of salvinorin A have been advanced. Positions C-1, C-2, C-4, C-17, C-18 and the furan ring itself have been altered (Harding et al., 2006; Harding, Tidgewell, Byrd, et al., 2005; Munro et al., 2005; Vortherms & Roth, 2006) (Fig. 10).

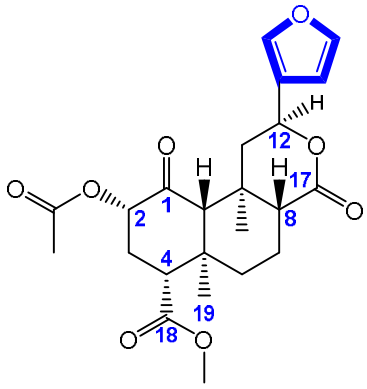


Fig. 10 - Salvinorin A chemical positions that have been submitted to modifications (blue colored)

It has been demonstrated that the C-2 position of Salvinorin A is one of the important binding sites to *kappa* opioid receptors. In order to formulate new drugs, with clinical purposes and better tolerance and *in vivo* stability, some modifications in this carbon have been made. For example, the synthesis of C-2 analogs like methoxymethyl ether and ethoxymethyl ether exhibit higher binding affinity and potency than salvinorin A to *kappa* opioid receptors. The first one also displays longer duration of action (Beguin et al., 2006; Lee et al., 2010). N-methylacetamide and 2-epi-isopropylamine derivatives, which provide increased stability and aqueous solubility are full agonists at KOR, having comparable strength to salvinorin A (Vortherms & Roth, 2006). Other synthetic analogues of salvinorin A, salvinorinyl-2-propionate and heptanoate derivative, revealed partial agonist activity at KOR's. Nevertheless, either heptanoate or propionate derivatives have much lower KOR affinity than salvinorin A (Chavkin et al., 2004). It is important to report that the C-2 position can only be occupied by small (3-4 carbons) lipophilic groups (Beguin et al., 2006; Chavkin et al., 2004; Lee et al., 2010). Lacking hydrophobic groups at such important binding position as C-2 (occurring the same at C-18) will result in a decreased affinity for kappa opioid receptors (Li et al., 2007). Given the fact that

halogen binding in biomolecular systems have gained wide acceptance, C-2 fluoro – , bromo- , cloro- and iodo- analogs of salvinorin A have been designed and evaluated about their affinities to μ-opioid receptors (MOR), δ-opioid receptors (DOR) and KOR. Some results have been observed: C-2-β isomer generally binds better than α isomer (with exception of iodinated analogs), and the affinity to the kappa opioid receptors increases with atomic mass (I > Br > Cl > F) (Lee et al., 2010). It is interesting to note that the decrease of affinity for *kappa* receptors caused by some of the changes in C-2 position (for example the introduction of an aromatic group), coincides with an increased affinity for μ-receptors (Harding et al. 2005; Tidgewell et al., 2006).

Changes at C-4 position suggested that the methyl ester group is essential for kappa opioid receptors activity (Lee et al., 2006). Nevertheless, changes in C-4 position are better tolerated than changes in C-2 position, since the derivates do not suffer an affinity decrease so evident (Tidgewell et al., 2006). Meanwhile, C-18 replacements by dimethylamide derivatives, esters, amines and ethers diminishes the affinity for KOR as well as the reduction induced in the C-1 ketone to a hydroxyl or acetoxyl group. Modifications made at the furan ring (experimentally replaced by N-sulfonylpyrrole, triazole analogs or oxadiazole analogs) and C-8 position led to a dramatic low level affinity for the KOR, at about ninetyfold less affinity and seventyfold less affinity, respectively. The obtained results, mainly enabled to realize that the methyl ester at C-4 and the furan ring at C-12 are fundamental for activity at KOR, while C-17 lactone is not so important (Harding et al. 2005; Munro et al., 2005; Yang et al., 2009).

Modifications and substitutions induced at the C-1 ketone of salvinorin A alter the agonist potential of the drug at the *kappa* opioid receptors. Therefore, analogues that have not the C-1 ketone, bind to opioid receptors in a different manner, producing an antagonist behavior, instead the typical agonist behavior (Holden et al., 2007).

Besides the fact that oxadiazole analogues disturb the connection and affinity for the KOR, substitution of the furan ring with a 4-methyl-1,3,5-oxadiazoline ring was identified as the first neoclerodane diterpene with kappa antagonist activity alongside with salvidivin A (synthetically obtained by photo oxidation of salvinorin A) (Simpson et al., 2007).

A careful and individual analysis to each target compounds of the molecular structure of salvinorin A, revealed the absence of any kind of bound with μ or δ opioid receptors. This result shows that salvinorin A will withstand considerable modification without losing its selectivity, since it will not establish any connection with other receptors besides KOR. The

affinity of the molecule might be decreased, but its selectivity will always be maintained (Munro et al., 2005).

It has been attributed to the furan ring of salvinorin A its earlier mentioned toxicity in reproductive system. Even recognizing the importance of this structure to bind receptors, many attempts were performed to mimic the furan ring at its binding site (Lovell et al., 2012). Most of the replacements resulted in less affinity for the *kappa* opioid receptors. Yet, replacing the furan ring by a piperidine ring or a thiomorpholine ring led to the formation of a selective partial agonists at *kappa* opioid receptors (although having less potency than salvinorin A) (Simpson et al., 2009).

3.6 Methods for identification and quantification of Salvinorin A in different matrices

3.6.1 – Detection of salvinorin A in plant material and commercial herbal products

3.6.1.1 - Thin Layer Chromatography (TLC)

TLC, although used for decades, continues to represent an important tool for the screening of compounds from vegetable origin, including drugs of abuse. Besides being a technique commonly adopted in the identification of methamphetamine, heroin and cocaine, it has already proven to be valid to separate alkaloids in *Salvia divinorum* leaves. TLC has shown ability to detect salvinorin A from extracted plant material and to easily distinguish *Salvia divinorum* from other *Salvia* species as well as from *Cannabis sativa L.* (Jermain & Evans, 2009; Kennedy & Wiseman, 2010) (Table I).

Kennedy and Wiseman (2010) developed a simple thin layer chromatography method combined with Desorption Electrospray Ionization-Mass Spectrometry (DESI-MS) that enabled salvinorin A detection in *Salvia divinorum* leaves. Besides the simplicity and inexpensive characteristics of TLC, this procedure combined the robustness of TLC with the sensitivity and specificity of mass spectrometry. Moreover, Siebert (2004) showed the high sensitivity for detecting salvinorins of TLC technique and, after preliminary experimentations, it was determined that salvinorin A can be detected in a 0.002% concentration solution. In a similar study, Jermain and Evans (2009) successfully extracted salvinorin A from commercial *Salvia divinorum* extracts (5X, 10X, 20X) using a chloroform:methanol mixture. The detection of salvinorin A, B, C, D and G in *S. divinorum* was performed by TLC. By using this technique, it was also possible to differentiate *Salvia divinorum* from other 13 *Salvia* species (Table I).

TLC has also been used for the localization of salvinorins in the plant. The method consisted in using chloroform swabs to extract salvinorins and evaluate the distribution pattern in leaf, steam, rachis, bract, calyx and corolla of mature plants. While the roots, internal stem tissue, cotyledons and corolla showed absence of salvinorins, the glandular trichomes of the abaxial, young leaves displayed the highest salvinorin content (Siebert, 2004) (Table I).

3.6.1.2- Liquid Chromatography

Gruber (1999) successfully developed a high performance liquid chromatography method with UV detection for salvinorin A identification and quantification, in leaves and stems of *Salvia divinorum*. A reverse phase C-18 column was used as stationary phase and the mobile phase was acetonitrile:water (45:55) in isocratic elution. Salvinorin A eluted in approximately 8.0-8.1 minutes and levels of salvinorin A in leaves ranged from 0.89 to 3.70 mg/g dry weight (Table I).

Later, Medana et al. (2006) used liquid chromatography coupled with electrospray ion trap mass spectrometry (LC/ESI-IT-MS) to characterize *Salvia divinorum* leaves. This method waived the eventual high pH value that would lead to hydrolysis of the ester group. The ion fragmentation, separation and quantification of six different salvinorins (A-F) and three divinatorins (A-C) were described. Salvinorin A limit of detection using LC/MS was 3 ng/mL (Table I).

Wolowich et al. (2006), in a similar study, investigated the content of five herbal products containing *Salvia divinorum* sold in the Internet or in several smart shops. The analysis was performed by high-performance liquid chromatography, but also by thin-layer chromatography and gas chromatography-mass spectroscopy, in order to detect other compounds, besides salvinorin A. Interesting results were obtained: salvinorin A concentrations were much lower than those claimed on the product label (1-16% of the claimed amount), and substances like vitamin E and caffeine were surprisingly found (Table I).

Another study attempted to determine and quantify salvinorin A and salvinorin B in products circulating in Japan. The experimental protocol relied in high performance liquid chromatography with ultraviolet (UV) detection. The extraction solvent selected was acetonitrile because the extracts were cleaner than those obtained with methanol and acetone, and also presented high extraction efficiency (Tsujikawa et al., 2008) (Table I).

3.6.1.3 - Gas Chromatography

Barnes and Snow (2012) used GC-MS to identify and quantify Salvinorin A and other alkaloid molecules in *Salvia divinorum* plant. For that purpose, liquid-liquid extraction (namely with chloroform) and solid-phase micro extraction were performed in several tissues of *Salvia divinorum* plant (roots, stems and leaves), followed by analysis and identification in GC x GC-

ToFMS (Gas Chromatography x Gas Chromatography – Time of Flight Mass Spectrometry). The methodology provided chromatographic separation of the closely related salvinorin analogs, being detected salvinorin A in stems and leaves, and salvinorin B and C only in leaves. Quantification was performed by using the characteristic fragments (m/z) of each molecule. Considering salvinorin A, the selected m/z ions were m/z: 94 (base peak), m/z: 166, m/z: 273, and m/z: 432 (molecular ion of salvinorin A) (Giroud et al., 2000) (Table 1).

Jermain and Evans (2009) analysed salvinorin A by GC/MS, performing the extraction from leaves with chloroform (when using a nonpolar solvent) and acetone (when using a polar solvent). Methanol was also tested, however a large amount of contamination chromatographic peaks was observed in the chromatogram, impairing the analysis. Combining an extraction of acetone or chloroform with GC/MS analysis, it was developed a fast procedure to evidence the presence of salvinorin A in plant material, which could be used for forensic purposes (Table 1).

Willard et al. (2012a) analyzed *Salvia divinorum* and four other *Salvia* species by GC/MS. All species samples were submitted to an extraction with dichloromethane. Due to the presence of salvinorin A, visual assessment of the chromatograms allowed the distinction of *Salvia divinorum* from the other species. Still, total ion chromatograms were submitted to principal component analysis (PCA) to provide a more objective comparison. Visual assessment of PCA scores plot allowed a clearer identification of *Salvia divinorum*. Also multiple procedures as Euclidean distances, Student's *t* test and hierarchical cluster analysis were performed (Willard et al., 2012a, 2012b). This methodology can be important to proofs to be presented in court (Table 1).

3.6.1.4 - Molecular Methods

Murphy and Bola (2013) managed to distinguish *Salvia divinorum* plant material from other plants, through the analysis of two different DNA sequences, that turned out to be specific of *Salvia divinorum*. The study of the sequence of the polymerase chain reaction of the ribulose bisphosphate carboxylase large subunit gene, enabled the differentiation between *Salvia divinorum* and other similar plants such as *C. sativa* and *N. tabacum*, whereas the study of DNA sequences adjacent to the chloroplast leucine transfer RNA gene, made possible to identify *Salvia divinorum* among other *Salvia* species.

Specific *Salvia divinorum* primers were designed on the sequence of the 5S-rRNA gene spacer region in order to develop Real-Time PCR detection strategies. This kind of procedures relies on continuous measurements of the increments in the fluorescence generated during the PCR. Real-Time PCR strategies present a broad dynamic range and low intra- and inter-assay variability (Luciano et al., 2007). Using this DNA fingerprinting method, resulting PCR products and subsequent alignment of the isolated nucleotide sequences of *Salvia divinorum* (about 500 bp) and *Salvia officinalis* (about 300 bp) exhibited great diversities in the spacer region of the two species. Additionally, a PCR- restriction fragment polymorphism (PCR-RFLP) was applied using restriction enzymes (*Nde*I and *Taq*I). Based on this PCR-RFLP method, *Nde*I site that was absent in *Salvia officinalis,* was found in *Salvia divinorum* NTS region at 428-433 bp and *Taq*I multiple sites found in *Salvia officinalis* (161-164, 170-173, and 217-220 bp) appeared in a unique site in *Salvia divinorum* (235-238 bp) (Bertea et al., 2006).

Along with other molecular methods, DNA barcoding has also already been used to detect *Salvia divinorum* in some products distributed in the internet as incense, and advertised as not for human consumption (Ogata et al., 2013).

3.6.2 – Detection of salvinorin A in human biological samples

3.6.2.1 – Liquid Chromatography

Analytical research of salvinorin A in body fluids has been object of study. A method using a solid-phase extraction technique coupled with liquid chromatography-electrospray ionization mass spectrometry gave promising results in blood and urine samples, with the limit of detection and limit of quantification standing respectively at 2.5 and 5.0 ng/mL (McDonough et al., 2008). This LC-MS methodology can be used in human biological fluid, however, it is important to sign that this method validation was conducted using certified drug-free urine instead of certified drug-free blood. Because half-life of salvinorin A is relatively short, and the dosage of the drug is very low, the blood sample would have to be collected almost immediately after exposure (Table 2). This implies that urine should be the biological sample indicated for legal purposes.

Schmidt et al. (2005) performed analytical procedures that coupled negative ion liquid chromatography with mass spectrometry/atmospheric pressure chemical ionization in order to determine *Salvia divinorum* compounds in body fluids, mainly plasma, cerebrospinal fluid and

urine. This method, with a high sensitivity, allowed the identification of salvinorin B as a metabolite of salvinorin A in biological fluids. The drawback of this method was that the samples were *ex vivo* spiked with salvinorin A, rather than taken after the systemic administration of the compound, thus, it was an implementation method, performed by spiking human fluid samples with the compounds (Grundmann et al., 2007) (Table 2).

3.6.2.2 – Gas Chromatography

A method to quantify salvinorin A in urine, saliva and sweat, using gas chromatography/mass spectrometry was developed by Pichini et al. (2005). The analytes were extracted from biological matrices taken from two volunteers after smoking a fixed amount of plant material. Samples were extracted with chloroform:isopropanol (9:1, v/v) and the concentrations found ranged between 0.015 and 5 μg/mL in urine and saliva and between 0.01 and 5 μg/patch in sweat. Recoveries ranged between 77.1 and 92.7% in the different biological matrices. The high drawback of this work was the number of samples: only two volunteers were involved in this study. Furthermore, it was not possible to have blood samples as the subjects refused blood collection after having smoked the plant leaves (Grundmann et al., 2007) (Table 2).

Besides the analysis of plant material, the already mentioned research of Barnes and Snow (2012), also applied GC x GC-ToFMS to analyze biological human samples, namely urine. The method was able to detect salvinorin A, but the most important conclusions were about the extraction procedure, which involved Solid-Phase Microextraction (SPME). SPME provided a better quantitative performance, with lower detection limit, thus being the most appropriate for physiological or clinical samples. Hence, Liquid-Liquid Extraction (LLE) represented a better alternative for researches on plant material, since it was more effective for higher concentrations (Table 2).

Table 1 - Published articles, about Salvinorin A detection and *Salvia divinorum* characterization using plant material or commercialized products with *Salvia divinorum*

Analytical Procedurehttp://www.youtube.com/watch?v=9z4Kft47Kbm	Sample	Extraction Techniques	Analytical figures	Main Results	References
GC X GC-ToFMS Stationary Phase: Two columns were used. The first one was a Phenomenex column 15m x 0.25mm, with 0.25µm of inner diameter. The second column was an Agilent Technologies column 1.5m x 0.25mm, with 0.25µm of inner diameter Flow rate: 1 mL/min	*Salvia divinorum* **plant tissues** (leaves, roots and stems)	**LLE:** Solvents: Water and chloroform	Calibration curve with linear range from 120 to 8000 ng/g	The highest quantity of salvinorin A was found in leaves. Nevertheless, only 60ng/g were obtained from the same tissue. Salvinorin B and salvinorin C were the most prevalent analogs of salvinorin A found	Barnes *et al.*, 2012
TLC –DESI: 2.5 x 7.5 cm sílica gel 60 F254 pre-coated TLC plates (250µm)	*Salvia divinorum* **leaves**	Extraction from the dry leaves in Acetone (1:5)	Linear Range: 1 to 7 mg/mL R^2 = 0.9998	Development of a simple and robust method that successfully extracted salvinorin A	Kennedy *et al.*, 2010
TLC: Silica Gel Plates Whatman 250µm.	Comercialized products with *Salvia divinorum* **leaves**	Extraction from the dry leaves in Methanol: Chloroform (1:1)	-	It was able to detect salvinorin A, B, C, D and G and to differentiate *Salvia divinorum* from other 13 *Salvia* species	Jermain *et al.*, 2008

TLC: Silica Gel Plates Whatman 250µm	*Salvia divinorm* **plant material**	Extraction was performed with 1 mL of chloroform for 100 mg of each sample	Lower limit of detection: 0.002%	It was able to clearly identify salvinorins A, B, C and D and to conclude that the neoclerodane diterpenes from Salvia divinorum are secreted as components of a complex resin that accumulates in peltate glandular trichomes	Siebert, 2003
GC-MS: Stationary phase: J&W Scientific HP-5 (5% phenyl-methyl-siloxane) capillary column (15m x 0.25m, with an inner diameter of 0.25µm) Carrier gas: helium Mass spectra collected in scan mode in the range of m/z 40-450	Comercialized products with *Salvia divinorum* **leaves**	Procedures of extraction tested: - Boiling chloroform for 10 minutes; - Extraction with methanol at ambient temperature; - Extraction with chloroform at ambient temperature	-	The authors concluded that, when the analysis is performed in GC/MS, chloroform (when using a nonpolar solvent) and acetone (when using a polar solvent) are the best solvents to achieve extraction	Jermain *et al.*, 2008
HPLC with UV detection (210nm) for quantitative analysis of salvinorin A **LC-MS** for quantitative analysis of salvinorin B Chromatographic separation performed with a Mightysil RP-18 column (2.0mm x 150mm, 5µm) Mobile phase: 0.05%formic acid in water and acetonitrile. Gradient analysis, with the following acetonitrile pecentages: 40%, 40-70%; 70-100%	Comercialized products with *Salvia divinorum* **leaves**	Extraction with acetonitrile (twice), followed by decoloration with graphite carbon powder	Linear range for salvinorin A: 50-2000ng R^2=0.993 Linear range for salvinorin B: 0-50ng R^2=0.993	The authors successfully identified and quantified salvinorin A and salvinorin B, from several different commercialized products	Tsujikawa *et al.*, 2008

100%; 40%.					
HPLC/UV-MS: Chromatographic separation performed with a Luna® Phenomenex column (2.0mm x 150mm, 3µm) Gradient mobile phase composition: 80/20 to 0/100 v/v water with 0.05% of formic acid/acetonitrile UV detector: 200-400nm **GC-MS:** Chromatographic separations ran in a Restek 5Ms column (30 m x 0.25 mm) Mass Spectra collected in full-scan mode in the range m/z 100-650	*Salvia divinorum* **leaves**	Extraction was previously performed with acetonitrile/water (50:50)	Limits of detection for LC/MS^2: Salvinorin A: 3ng/mL; Salvinorin B: 7ng/mL; Salvinorin C: 2ng/mL; Salvinorin D: 9ng/mL. Limits of Detection for GC/MS: Salvinorin A: 40ng/mL; Salvinorin B: 51ng/mL; Salvinorin C: 46ng/mL; Salvinorin D: 36ng/mL; Limits of Detection for LC/UV (220+288 nm): Salvinorin A: 367ng/mL; Salvinorin B: 1144ng/mL; Salvinorin C: 221ng/mL; Salvinorin D: 128ng/mL	The study allowed the study of nine different diterpenes. LC-MS revealed to be a particular useful technique for the analysis of the herbal products containing *Salvia divinorum*	Medana *et al.*, 2006

			Linear range: 10-5000ng/mL		
HPLC: Stationary phase: Zorbax 300 SB-C18 column (250m x 4.6mm, 5µm); Mobile phase: acetonitrile:water (45:55) Salvinorin A detection: UV 208nm	Comercialized products with *Salvia divinorum* **leaves**	Extraction performed with chloroform for 30 min	Correlation Coefficient (r^2): 0.9998	Salvinorin A concentrations were much lower than those claimed on the products labels. Substances such as vitamin E and caffeine were surprisingly found	Wolowich *et al.*, 2006
HPLC: Stationary phase: Zorbax 300 SB-C18 column (250m x 4.6mm, 5µm); Mobile phase: acetonitrile:water (45:55) Salvinorin A detection: UV 208nm	Plant tissues (**leaves and stems**) of *Salvia divinorum*	Extraction performed with chloroform for 30 min	Correlation Coefficient (r^2): 0.9997	The authors managed to develop one of the first procedures to successfully extract salvinorin A from *Salvia divinorum* plant material	Gruber *et al.*, 1999
GC-MS: Stationary phase: DB-5MS column (30m x 0.25mm x 0.25µm); Carrier Gas: Helium 1 mL/min	*Salvia divinorum* **leaves**	5 min extraction with dichloromethane	Correlation coefficient (r^2): 0.9981	It was possible to combine statistical procedures with GC/MS analysis, to improve the demonstration of differences between *Salvia divinorum* and other *Salvia* species	Willard *et al.*, 2012

GC-MS: Stationary phase: HP Ultra-2, 5% phenyl-methyl-silicone capillary column (25m x 0.2mm x 0.33µm); Carrier Gas: Helium 1.2 mL/min	Commercialized products with *Salvia divinorum* **leaves**	Liquid-liquid extraction using a mixture of chloroform and isopropanol (9:1, v/v). After drying the plant material it was mixed with methanol and then dissolved in acetonitrile	-	This study represented one of the first successfully attempts to extract salvinorin A with GC/MS. It was possible to detect the major peaks at m/z: 94, m/z: 166, m/z: 273, and m/z: 432	Giraud *et al.*, 2000

Table 2 - Published articles, about Salvinorin A detection and *Salvia divinorum* characterization in biological matrices

Analytical Procedure	Sample	Extraction Techniques	Analytical figures	Main Results	References
GC X GC-ToFMS Stationary Phase: Two columns were used. The first one was a Phenomenex column 15m x 0.25 mm x 0.25µm. The second column was an Agilent Technologies column 1.5m x 0.250mm x 0.25µm Flow rate: 1 mL/min	Human **urine** spiked with salvinorin A	LLE: Solvents: Water and chloroform SPME: Polyacrilate fiber 85 µm	Liquid-Liquid Extraction: LOD: 200 ng/mL. Linear range: 300-5000 ng/mL SPME: LOD: 4ng/mL. Linear range: 8-500 ng/mL	SPME range proved to be more appropriate for clinical and physiological samples. LLE was found more effective for higher concentrations that may be found in plant material or products containing salvinorin A	Barnes et al., 2012
LC-MS/APCI: Stationary phase: Phenomenex Polar-RP (150 mm x 2.0 mm x 4µm) Isocratic analysis: Solvent A: 4mM Ammonium Acetate (50%); Solvent B: Acetonitrile (50%)	Human Biological fluids (**Urine, Plasma**)	Solid phase extraction: Cartridges were washed with 10% methanol/water and dried with nitrogen. Analytes were eluted from cartridges with 75% dichloromethane in acetonitrile. Solvent was removed with nitrogen and samples ressuspended in 100 µl of 75% acetonitrile/water	LLOQ:2ng/mL Linear Range: from 2 to 1000 ng/mL	The method allowed the identification of salvinorin B as a metabolite of salvinorin A in biological fluids	Schmidt et al., 2005

LC-MS: Chromatographic separation performed with a Luna® phenyl-hexyl column (Phenomenex, 2.1mm x 100mm, 5µm) Mobile Phase: 0.1% formic acid and acetonitrile Gradient analysis, with the following acetonitrile percentages: 40%; 75%; 90%	Human **Urine**	Elution of the biological fluids with 25% of acetonitrile in dichloromethane	Linear range for salvinorin A: 5.0-100 ng/mL R2=0.997; LOD: 2.5ng/mL; LOQ: 5.0ng/mL;	The authors a method to successfully identify salvinorin A in human urine	McDonough et al., 2008
GC-MS: Stationary phase: Fused-silica capillary column (HP-5MS 30m x 0.25µm) Carrier Gas: High purity helium (99%) at flow rate of 1 mL/min	Biological Samples (**blood, urine, saliva and sweat**)	All the samples were submitted to liquid-liquid extraction with chloroform / isopropanol (9:1)	LOD for Plasma, Urine and Saliva: 0.005 µg/mL; LOQ for Plasma, Urine and Saliva: 0.015 µg/mL LOD for Sweat: 0.003µg/mL; LOQ for Sweat: 0.010 µg/mL Correlation Coefficients (r2): Plasma: 0.997; Urine: 0.999; Saliva; 0.996; Sweat: 0.999.	This study was the first one to detect salvinorin A in urine and saliva samples, only 1.5h after consumption. A simple GC/MS procedure was able to identify salvinorin A in various biological matrices	Pichini et al., 2005

Part II:

Experimental

1- Objectives

2- Materials and Methods

3- Results and Discussion

4- Conclusion

1- Objectives

According to Favretto et al. (2013), some of the most important challenges of the moment in Forensic Toxicology are the new psychoactive substances, and the challenge to develop methodologies that can effectively detect and quantify them. Those remarks legitimize the present thesis, in which it will be studied one of the most prominent drugs, *Salvia divinorum*, easily available in smartshops and via internet. Besides the hallucinogenic effects induced by *Salvia divinorum*, its legal purchase in many countries, greatly justify the growing interest of the scientific community.

In the present thesis, framed in the Master of Forensic Sciences, it was scrutinized the composition of 10 different samples of *Salvia divinorum*, that were, at the time of its purchase, legally sold on e-commerce and in "smart shops" in Portugal.

The specific objectives of this work are to validate a protocol of extraction of salvinorin A and other related molecules from concentrated extracts of *Salvia divinorum*; test different solvents of extraction and choose the best one; determine the chemical composition of the products, evidencing the presence of the only hallucinogenic compound of the plant (salvinorin A); quantify salvinorin A and estimate the amounts of salvinorins B, C and D; confront the obtained results with previous similar researches on *Salvia divinorum* commercialized products and evaluate the reliability and quality of the information that is provided to consumers in the packages of the products.

Besides the specific objectives mentioned above, it was also intended to provide a literature review on the state of the art of *Salvia divinorum*, contribute to a better knowledge of the drug and to encourage a responsible attitude from both consumers and marketers, thus protecting society in general.

2- Materials and Methods

2.1- Samples and Salvinorin A Standard

Salvia divinorum extracts were purchased from September of 2012 until April 2013. Ten samples were purchased in four different "smart shops": "Magic Mushroom" (Porto, Portugal), "Magic Mushroom" (Espinho, Portugal), "Cogumelo Mágico" (Aveiro, Portugal), "Euphoria" (Porto Portugal), and in 2 websites: http://azarius.pt/ and http://www.deliriumsmartshop.com (Fig.11, Fig.12). The samples are classified in "5x", "10x", "15x", "20x", "40x" or "60x" and the amounts of *Salvia divinorum* concentrated extracts were generally 1g. Many of the packages did not explain what this classification meant and if this is related to the salvinorin A contents. Only samples acquired from Azarius® guarantees through the above cited company's website, that for example the "5x" extract has 12.5 mg of salvinorin A, while the "10x" extract contains 25 mg of salvinorin A. Packages from Euphoria "smart shop" have printed in the label that one gram of the "5x" extract equals five grams of *Salvia divinorum* leaves; one gram of the "10x" extract equals ten grams of *Salvia divinorum* leaves, and so on. Other information provided to customers is displayed in table 3, but, in most of the cases only scarce information was provided.

After the purchase, all the samples were kept in a humidity controlled environment, at room temperature (similar to the environment observed in "smart shops").

Salvinorin A standard was purchased from Sigma-Aldrich Chemie Gmbh (Munich, Germany), and allowed the identification and quantification of salvinorin A in the samples. Salvinorin A standard was kept at -20°C, in a humidity controlled environment, and protected from artificial light or sun light.

2.2- Reagents

Acetonitrile and acetone were purchased from Fisher Chemical®, having 99.99% and 99.98% of purity respectively, according to GC assays. Chloroform was obtained from Fisher Scientific® and had 99.99% of purity, also determined by GC assay. Thymol, used as internal standard, was purchased from Sigma Aldrich®, and referred a purity ≥ 99.5%. All the other reagents were of the highest purity available but at least of analytical grade.

Fig. 11 - Packages of the acquired samples of concentrated extracts of *Salvia divinorum*

Fig. 12 - Example of the usual presentation of bags containing *Salvia divinorum* extracts, and usual appearance of the extract

Table 3 - Information available in every packages of concentrated extracts

Samples	Responsible for selling	Labeled Potency	Batch	Expiration	Weight	Additional information in packaging
"Salvia divinorum 10x" - Magic Mushroom	Magic Mushroom - Porto	10x	0b03	Jun-13 /Apr-13	1g	"Causes a state of unreal consciousness"; "Sensations similar to those obtained with marijuana"; "Use in a quite environment"; "Some people might experience slight headache, insomnia and bronchial irritation"; "Should be used in the presence of a sober person"; "Do not cause dependence"; "Not dangerous"; Historical Background
"Salvia divinorum Extrato" - Euphoria	Euphoria – Porto	5x	2129560	Feb-14	1g	Origin of the plant; "Forbidden to be purchased by minors"; "Provokes hallucinations, mind-body experience, travel to the past, becoming an object, presence in several places at once, and uncontrollable laughter"; "Do not smoke extracts stronger than 5x for the first time"; "Smoke in a bong or pipe"; "Do not cause dependence"; "Not dangerous"
"Salvia divinorum 40x" - Magic Mushroom	Magic Mushroom – Porto	40x	0d03	Jun-13	1g	Historical Background; Traditional use by *Mazatec* indians; Preparation: "Boil about 200mL of water for a cup of tea and add the sage"
"Sálvia divinorum 15x" - Cogumelo Mágico	Cogumelo Mágico – Aveiro	15x	?	Aug-14	0.5g	How to use ("smoke in pipe or bong, or prepare a tea"); "Product 100% natural"
"Sage Extract 10x" - Azarius.net	Azarius® website http://azarius.pt/	10x	?	?	1g	Origin of the plant; Effects ("hallucinations start in 10 minutes and last for 45 minutes"); Interactions ("Must be avoided simultaneous administration with alcohol, other drugs and monoamine oxidase inhibitors"
"Salvia divinorum 20x" - Deliriumsmartshop.com	"Delirium" website http://www.deliriumsmartshop.com	20x	0d03	Jun-13	1g	Historical Background; Traditional use by *Mazatec* indians; Preparation: "Boil about 200mL of water for a cup of tea and add the sage"
"Salvia divinorum 10x" - Magic Mushroom	Magic Mushroom - Espinho	10x	0b03	Jun-13	1g	"Causes a state of unreal consciousness"; "Sensations similar to those obtained with marijuana"; "Use in a quite environment"; "Some people might experience slight headache, insomnia and bronchial irritation"; "Should be used in the presence of a sober person"; "Do not cause dependence"; "Not dangerous"; Historical Background
"Salvia divinorum Extrato" - Euphoria	Euphoria – Porto	10x	?	?	1g	Origin of the plant; "Forbidden to be purchased by minors"; "Provokes hallucinations, mind-body experience, travel to the past, becoming an object, presence in several places at once, and uncontrollable laughter"; "Do not smoke extracts stronger than 5x for the first time"; "Smoke in a bong or pipe"; "Do not cause dependence"; "Not dangerous"; "Smoke

						salvia in a quiet, dark, environment"; "TV can destroy the experience, but appropriate music might be inspiring"
"Salvia divinorum Extrato" - Euphoria	Euphoria - Porto	40X	L1840	Feb-14	1g	Origin of the plant; "Forbidden to be purchased by minors"; "Provokes hallucinations, mind-body experience, travel to the past, becoming an object, presence in several places at once, and uncontrollable laughter"; "Do not smoke extracts stronger than 5x for the first time"; "Smoke in a bong or pipe"; "Do not cause dependence"; "Not dangerous"; "Smoke salvia in a quiet, dark, environment"; "TV can destroy the experience, but appropriate music might be inspiring"
"Salvia divinorum Extrato" - Euphoria	Euphoria - Porto	60X	L1860	Feb-14	1g	Origin of the plant; "Forbidden to be purchased by minors"; "Provokes hallucinations, mind-body experience, travel to the past, becoming an object, presence in several places at once, and uncontrollable laughter"; "Do not smoke extracts stronger than 5x for the first time"; "Smoke in a bong or pipe"; "Do not cause dependence"; "Not dangerous"; "Smoke salvia in a quiet, dark, environment"; "TV can destroy the experience, but appropriate music might be inspiring"

2.3- Experimental Conditions

Gas-Chromatography analysis was achieved using a Varian CP-3800 gas chromatograph (USA) equipped with a Varian Saturn 4000 Ion Trap mass selective detector (USA) and a Saturn GC/MS workstation software version 6.8. Stationary phase consisted in a capillary column VF-5ms (30m x 0.25mm x 0.25µm) from Varian (USA). Helium C-60 (Gasin, Portugal) at a constant flow rate of 1.0 mL/min, was used as mobile phase. Two microliters of each extract were injected using a split ratio 1:40 at 250 °C. The column oven temperature was maintained at 100 °C for 1 minute and then raised to 300 °C at 15 °C/min and hold at 300 °C for 20 minutes totaling 34.3 minutes. Trap setpoint was at 180 °C, manifold setpoint was at 50 °C, and transfer line setpoint was at 280 °C. Electron energy was 70Ev. In order to avoid solvent overloading, ionization was maintained off during the first 4 minutes. Data were collected from m/z 40-1000. The emission current was 30 µA and the maximum ionization time was 25000 µs. Mass spectra acquisition occurred between 4 and 34 minutes, after the injection of the samples.

All determinations were performed in Full Scan Mode. For quantification purposes full scan chromatograms were reconstructed through the selection of qualifier ions for each molecule. The selected ions used for the quantitative measurements were: *m/z* 94, *m/z* 273 and *m/z* 432 for salvinorin A; *m/z* 43, *m/z* 94, *m/z* 291, *m/z* 372 and *m/z* 390 for salvinorin B; *m/z* 94, *m/z* 313, *m/z* 372 and *m/z* 414 for salvinorin C; *m/z* 94, *m/z* 313, *m/z* 400 and *m/z* 432 for salvinorin D.

The identification of salvinorin A in samples was performed by comparison of retention time and mass spectrum of salvinorin A standard and salvinorin A in samples, under the same chromatographic conditions, and through the match probability obtained in SWG DRUG library of spectra. The remaining salvinorins in study (B, C and D) were identified comparing retention times and mass spectra of the peaks obtained upon the samples injection with previous published work and results obtained by Jermain and Evans (2009).

2.4- Extraction Procedure

To determine the most effective solvent of salvinorin A from *Salvia divinorum*, three solvents with different polarities were tested (acetonitrile, acetone, and chloroform) (Table 4). The main objective was to extract the greatest amount of salvinorin A, but it was also important to extract salvinorin B, C, D and other related compounds. All the solvents extraction was tested in triplicate.

Table 4 – Polarity indices of the three tested solvents

Solvent	*Polarity Index*
Chloroform	4.1
Acetone	5.1
Acetonitrile	5.8

2.4.1- Extraction with acetonitrile or acetone

Samples were submitted to a process of extraction already performed by Tsujikawa et al. (2008), with some modifications, as for example the addiction of an internal standard to avoid misleading conclusions because of eventual losses during the extraction procedure. Thus, all the samples were grounded in a mortar into a thin powder. Then, 50 milligrams of each sample was transferred to a tube. In this tube, 2 mL of acetonitrile/acetone and 0.1 mL of the internal standard (thymol, 1mg/mL) were added, followed by one minute vortex shaking and five minutes ultrasonication. The mixture was centrifuged at 3000 rpm for three minutes, being the supernatant transferred into a second tube. To the first tube, it was added 2 mL more of acetonitrile or acetone, and it was again shaken, ultrasonicated, centrifuged and transferred to the second tube, being both extracts combined. The extractions were performed in triplicate for each sample (Fig. 13).

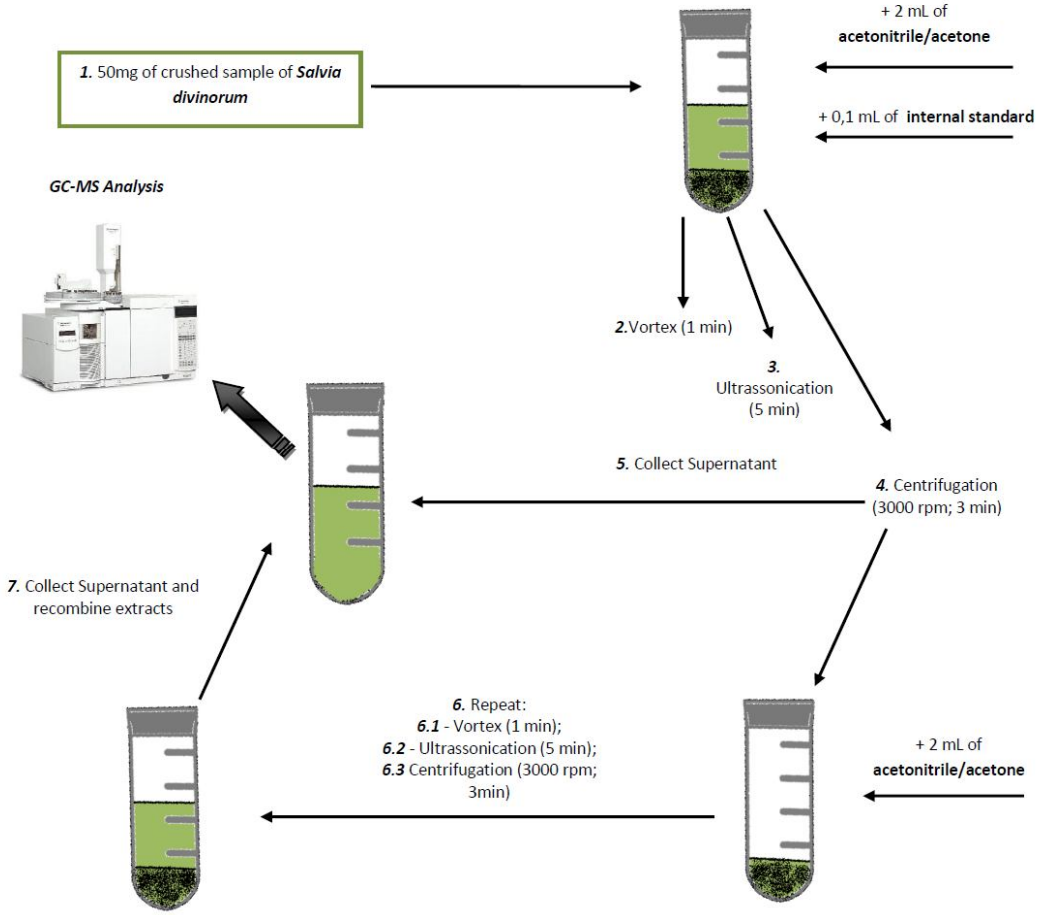


Fig. 13 - Extraction Procedure Performed with Acetone or Acetonitrile

2.4.2- Extraction with chloroform

In order to perform the extraction with chloroform, 50 mg of the tested samples were submitted to extraction with 4 mL of chloroform. At this stage, 0.1 mL of the internal standard (Thymol 1 mg/mL) was added. The mixture was vortexed for 1 minute and ultrassonicated for 5 minutes. Then, it was performed a filtration with a filter for organic solvents, thus separating the plant material from the concentrated solution (Fig. 14).

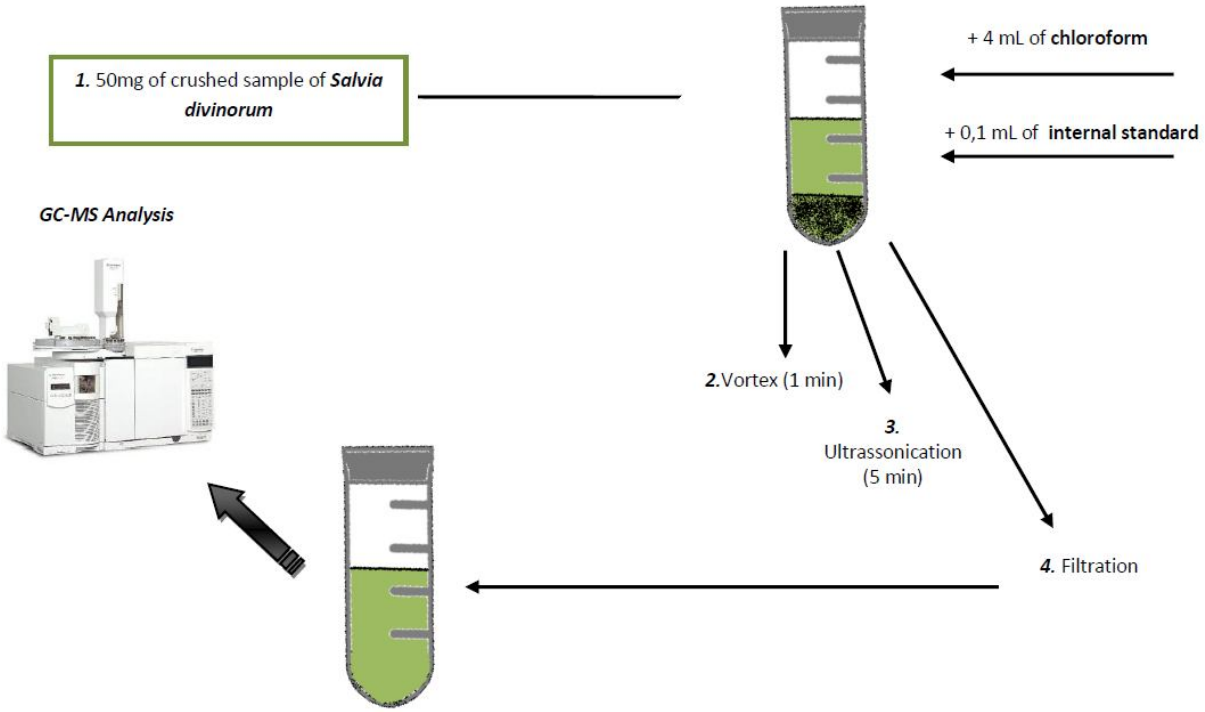


Fig. 14 - Extraction Procedure Performed with Chloroform

2.5- Concentrated Samples

One extract of each sample was concentrated in order to identify different compounds that could be present in very low concentrations, as well as to evidence the presence of salvinorins C and D, that are presented in small amounts (very small peaks in the chromatograms). To achieve this, 2mL were taken from the solution obtained by the extraction procedures cited above, and were concentrated by evaporation of the solvent with slight nitrogen current (Fig. 15).

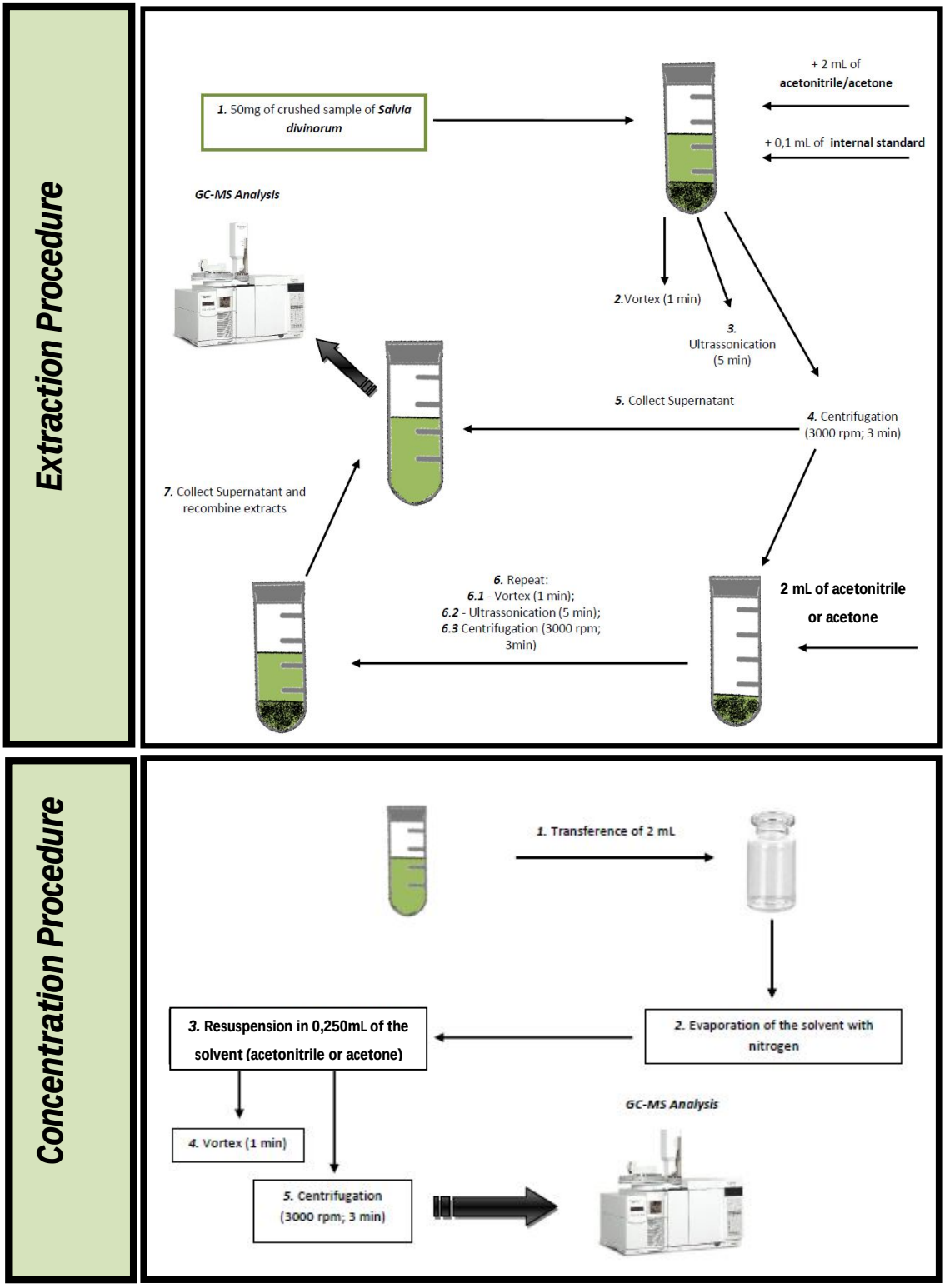


Fig. 15 - Example of the Concentration of the Samples after Extraction (in this case with most polar solvents)

2.6- Method Validation

According to Scientific Working Group for Forensic Toxicology (SWGTOX), the method validation may be defined as a process of conducting a series of experiments that estimate effectiveness and reliability of the analytical method, or modification of a previously validated analytical method. A method must be adequate for its purpose, and only validation may objectively evidence its reliability, by fulfilling a set of requirements, also proving its applicability. Although, establishing objectively that a method is capable of having a successful performance at the use for which it was developed, is not the only aim of validation. It is equally important to identify the limitations of the method under normal operating conditions (SWGTOX, 2013).

In the field of Forensic Toxicology, validation is fundamental, in order to ensure the legitimacy of a method. According to Peters et al. (2007), the impact of unsubstantiated results might be felt in the scientific community, as well as in society. Regarding the scientific community, the unsubstantiated results may lead to undervalued or overvalued effects, false interpretations and unreliable conclusions. Relatively to forensic field, unsubstantiated results might not withstand the scrutiny of a court, where gathering objective evidence is primordial, or even worse, bring unfair consequences for the defendant, eventually contradicting the principle of *in dubio pro reo*.

One of the most frequent concerns, regarding *Salvia divinorum* analytical studies, is the identification and quantification of its only hallucinogenic compound, salvinorin A. Taking into account the existence of few studies validated for the detection of salvinorin A, and the need to optimize the procedures, making them more effective and robust for forensic purposes, researchers continue to look for the best extraction procedure and the best analytical technique. As one of the objectives of this work was to validate a procedure that could identify and quantify mainly salvinorin A, but also salvinorins B, C and D, several solvents were tested and different extraction protocols were experimented.

The presence of salvinorin A in samples was evidenced by comparing the mass spectrum of Salvinorin A in samples with those existing in SWGDrug and National Institute of Standards and Technology (NIST) 05 mass spectra libraries. It was also confirmed by using salvinorin A standard, which makes possible the quantification of this compound in samples. Regarding the identification of salvinorins B, C and D, standards were not available, therefore retention time and characteristic mass spectra from Jermain and Evans (2009) works were

taken into account. In order to quantify salvinorins B, C and D, the calibration curve obtained from the salvinorin A standard was conveniently adopted, being results presented as semi-quantitative.

To improve the quantitative analysis precision, an internal standard was added. Thymol (1mg/mL) was chosen since it is highly soluble in organic solvents, and is well separated from the other compounds present in samples. According to the Society of Forensic Toxicologists and the Toxicology Section of the American Academy of Forensic Sciences (SOFT-AAFS), the introduction of an internal standard is recommended for all chromatographic assays (GC, HPLC). The internal standard must have physical and chemical properties similar to those of the analyte, and shall be added at one of the earliest stages of the procedure, if possible, in the extraction process (SOFT/AAFS, 2006).

The extraction procedure and the analytical technique were submitted to validation criteria required by the European Medicines Agency (EMEA) (EMEA, 2011), Food and Drug Administration (FDA) (FDA, 2001), and, according to the parameters that are commonly accepted as indispensable in quantitative and qualitative bioanalytical procedures such as: selectivity, linearity (model of calibration), limits of detection (LOD) and quantification (LOQ) and precision (repeatability).

2.6.1- Linearity

The construction of a calibration model is important to investigate the relationship between the concentration of the analyte in sample and the corresponding reply, which is, in this case, the area of the peak. According to most of the guidelines, 5 levels of concentration of the analyte is the minimum acceptable (Peters et al., 2007). Furthermore, the concentrations should cover the entire range of concentrations in the extracts to be analysed. The concentrations of the standard of salvinorin A that enabled the establishment of a calibration curve were: 0 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 500 μg/mL and 1000 μg/mL. It was added 40 μL of the internal standard in each solution.

In order to quantify salvinorins B, C and D, and in the absence of standards of these compounds, the calibration curve obtained for salvinorin A was conveniently adapted. Given the much lower values of the signals of these compounds, the quantity of salvinorin B was estimated with the calibration curve constructed with the following concentrations of

salvinorin A: 0 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, and for the quantification of salvinorins C and D the concentrations of 0 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL, 50 μg/mL, 100 μg/mL of salvinorin A standard were used.

The method linearity was evaluated by the square correlation coefficient obtained for each calibration curve (one for salvinorin A, one for salvinorin B and one for salvinorins C and D). SOFT-AAFS considers that, for most applications, 0.99 is considered an acceptable correlation coefficient. Only in certain circumstances, 0.98 might be considered a value minimally acceptable (SOFT/AAFS, 2006).

2.6.2- Precision

In order to evaluate the method's repeatability, it was determined intra-day and inter-day precision. Mean, standard deviation and coefficient of variation were determined. According to Food and Drug Administration (FDA) (FDA, 2001) , the precision determined at any concentration level should be equal or less to 15% of the coefficient of variation.

2.6.2.1- Intra-day precision

In order to verify intra-day precision, it was performed 5 different aliquots of one same sample in the same day. The sample was held from a mixture of the different original samples. An amount of 50.0 mg from the referred mixture was extracted with 4 mL of acetonitrile (the concentration of each of the aliquots was 1.25 mg/mL), according to the established procedure. It was calculated the mean of the samples, as well as standard deviation and coefficient of variation. All injections were performed by the same operator and analyzed by the same apparatus.

2.6.2.2- Inter-day precision

Aiming to evaluate if the method was reproducible in different days, portions of 50.0 mg of a mixture of different samples were submitted to analysis. Days 1, 3 and 4 were evaluated. On each day, five different amounts of 50.0 mg were extracted with 4 mL of

acetonitrile (the concentration of each of the new sample was 1.25 mg/mL), according to the established procedure. The means of salvinorins A, B, C and D of the three different days were compared, as well as the standard deviation and coefficient of variation. All extracts and injections were performed by the same operator and analyzed in the same apparatus.

2.6.3- Sensitivity

The limit of detection (LOD) expresses the minimum concentration of the analyte that can be detected, but not necessarily quantified, or the lowest concentration of the analyte in sample, that can reliably be differentiated from background noise (Peters et al., 2007). In order to determine the value of LOD, several methods might be implemented. One of the most common is the signal-to-noise ratio (S/N). When the determination is about the limit of detection, a S/N equal or greater than 3 is considered acceptable (SOFT/AAFS, 2006).

The lower limit of quantification (LLOQ) represents the lowest amount of an analyte in a sample that can be quantitatively determined (Peters et al., 2007). So it can be determined with precision, one of the eligible methods, is also the signal-to-noise ratio. However in this case, it is usually required a S/N equal or greater than 10 (SOFT/AAFS, 2006).

LOD and LLOQ concentrations were tested in quadruplicate, being the means, standard deviations and coefficients of variation taken into account.

3- Results and Discussion

3.1- Selection of the solvent

In order to select the best solvent among acetonitrile, acetone and chloroform, a mixture of several samples was prepared to test the extraction efficiency of the three solvents. The extraction of any of the solvents was performed in triplicate.

Table 5 – Peak Areas of salvinorins resulting from extractions with different solvents

Different Solvents	SalA		SalB		SalC		SalD	
	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
Acetone	1.33E+07	6.20E+05	6.60E+06	3.47E+05	6.31E+05	5.24E+04	6.85E+04	4.41E+04
Acetonitrile	1.34E+07	1.42E+06	5.33E+06	7.67E+05	5.52E+05	7.07E+04	4.30E+04	2.09E+04
Chloroform	1.21E+07	2.21E+06	5.96E+06	5.06E+06	5.72E+05	1.56E+05	?	?

All the solvents managed to extract salvinorins A, B and C, but the identification of salvinorin D was not possible using chloroform (Table 5).

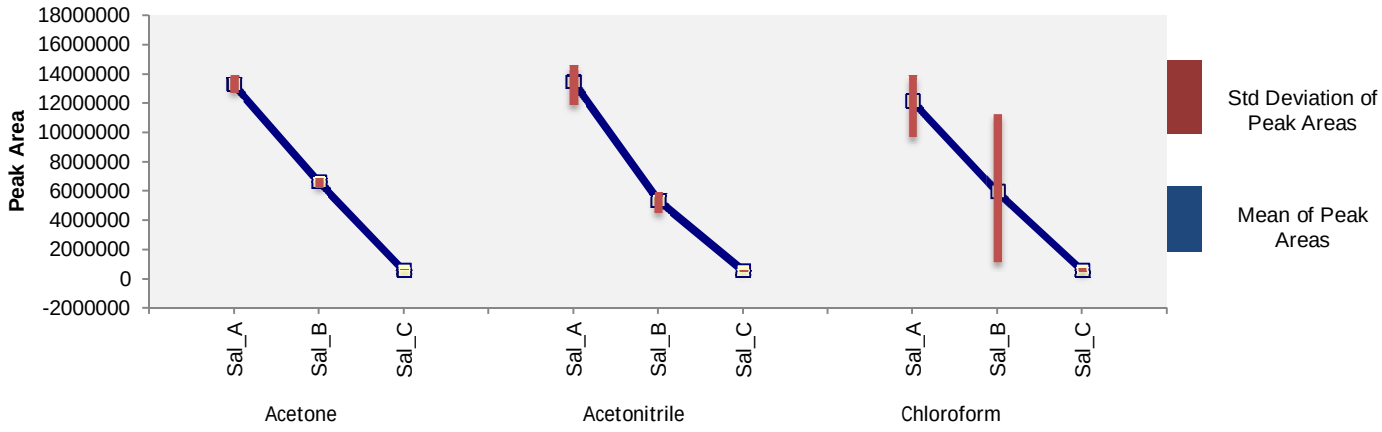


Fig. 16 - Extractions of salvinorins A, B and C, with different solvents

Regarding the extraction procedure, Jermain and Evans (2009) referred that the best solvents to extract salvinorin A from *Salvia divinorum* samples were acetone or chloroform. However, this study lacked the comparison between the mentioned solvents with acetonitrile. Adopting the extraction procedure designed and validated by Tsujikawa et al. (2008) for both polar solvents tested, acetone and acetonitrile, the efficiency of extraction of salvinorin A was very similar, being acetonitrile slightly more effective, since the peak areas had higher values. Nevertheless, acetone revealed a homogenous extraction value between the assays, thus having a lower standard deviation and a higher precision (Fig. 16). As for the peaks shape, they were very similar, having a good resolution in both cases. With regard to extraction with chloroform, the mean of the peak areas of salvinorin A was much lower than the results obtained with acetonitrile. Nevertheless, once again, the resolution of the peaks was acceptable. Besides being the solvent with the lower extraction efficiency of salvinorin A, chloroform also had the disadvantage of not allowing the identification and quantification of salvinorin D in 2 of 3 trials. In addition, although the majority of crime laboratories usually accomplishes the extraction with chloroform (Jermain & Evans, 2009), the separation between the two phases is worst after centrifugation, than with other solvents. In fact, with chloroform, it is necessary to perform a filtration to remove the plant solid residue. The filtration process is fairly lengthy because of the large amount of plant material that eventually saturates the filter, implying its constant renewal. Admitting the importance of a fast recognition of the drug in forensic laboratories, this represents a relevant disadvantage.

The extractions of salvinorins B, C and D were more efficiently performed using acetone as solvent. Nevertheless, only in the case of salvinorin B, the difference between the extraction performed with acetonitrile and acetone was substantially different.

Towards the results obtained, the extraction with acetonitrile was the preferred. It was also considered results obtained from Tsujikawa et al. (2008) evidencing that acetonitrile allowed a best rate of extraction.

3.2- Method Validation

3.2.1- Linearity

Linearity studies were performed using salvinorin A standard ranging from 5 μg/mL to 1000 μg/mL to quantitatively determine salvinorin A, whereas concentrations between 5 μg/mL and 200 μg/mL were used to perform a semi-quantitative determination of salvinorin B. The selected range to semi-quantitative determine salvinorins C and D was 5-100 μg/mL (Table 6).

Table 6 - Values for Calibration Curve - Linearity

A
B
C; D

Concentration (μg/mL)	Ratio between peak areas (sal A/Int Std)
0	0
5	0.03
10	0.11
20	0.13
50	0.37
100	0.76
200	1.36
500	2.59
1000	5.11

The correlation coefficients obtained from the 3 calibration curves were above 0,99, being therefore acceptable according to SOFT/AAFS (2006) (Fig. 17-19). After the insurance of linearity of the defined models, equations of the lines made possible to determine the concentrations of the different studied salvinorins, in which "x" corresponds to the concentration of the analyte, and "y" is the value of the correspondent peak area ratio (sal A / Internal Standard) (Table 7).

Table 7 - Calibration models for salvinorins A-D

Compound(s)	Equation of the line	Range (µg/mL)	R^2
Salvinorin A	*y= 0.05x + 0.1049*	0-1000	0.9951
Salvinorin B	*y= 0.0069x + 0.017*	0-200	0.9958
Salvinorins C and D	*y= 0.0076x + 0.0004*	0-100	0.9962

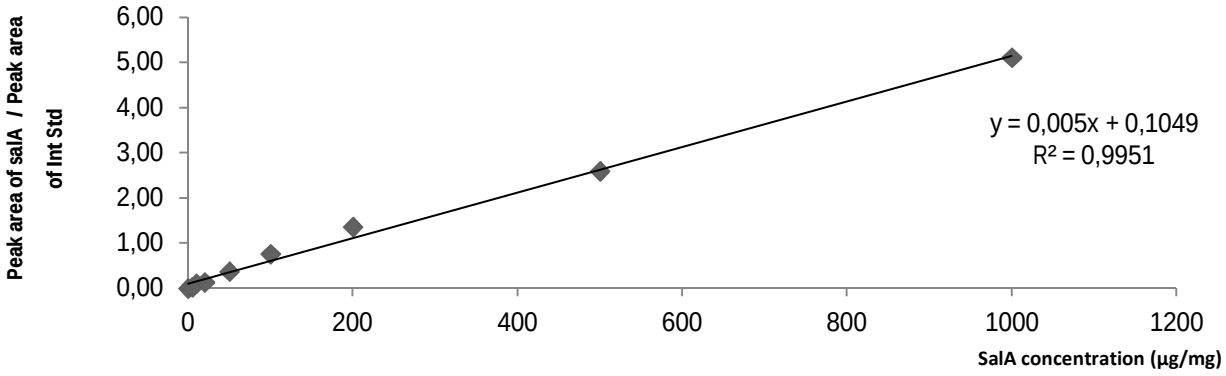


Fig. 17 - Calibration curve of salvinorin A

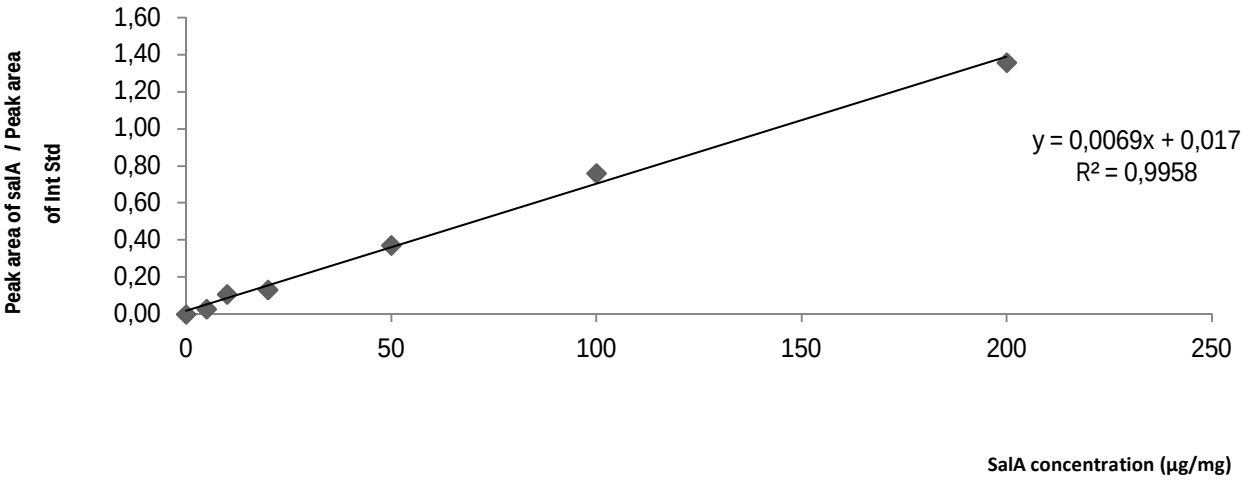


Fig. 18 - Calibration curve for salvinorin B

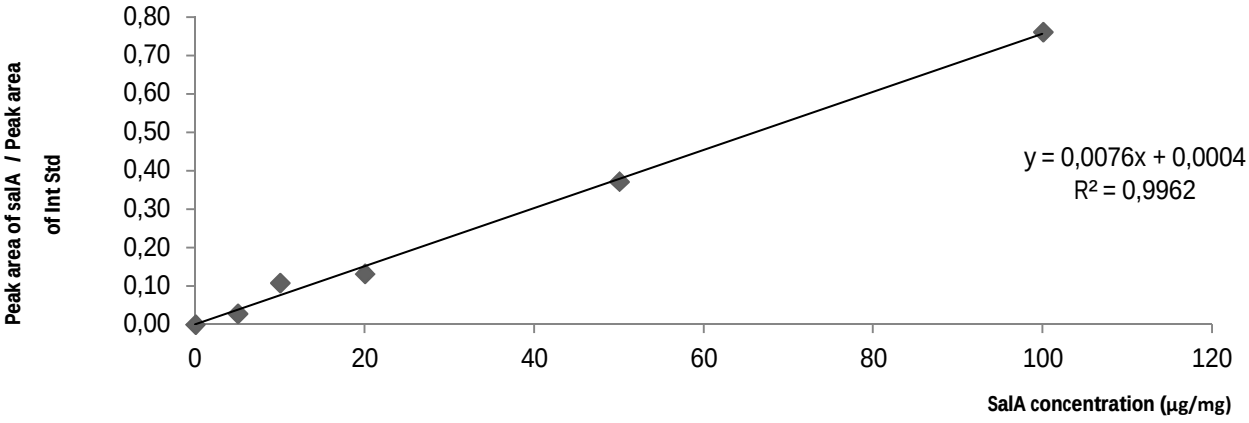


Fig. 19 - Calibration curve for salvinorins C and D

3.2.2- Precision

The obtained results in intra-day and inter-day analysis revealed the precision of the adopted procedures of extraction and analysis of salvinorins A, B and C, since the intra-day (n=5) coefficient of variation was between 3.63% and 8.60%, and the inter-day coefficient ranged from 6.64% to 14.88% (n=3) (Tables 8, 9). Although the intra-day coefficient of variation of salvinorin D revealed an adequate value of 9.22%, inter-day coefficient exceeded the 15% limit admitted by U S Food and Drug Administration (FDA, 2001) (Table 9). Extremely low concentrations of salvinorin D turned out to be a barrier to the semi-quantitative determination of the compound, revealing an inter-day precision of 18.22%.

Table 8 - Intra-day Precision (n=5)

Compound	Mean (mg/g)	Standard Deviation(mg/g)	Coefficient of Variation (%)
SalA	785.90	28.55	3.63
SalB	332.54	28.59	8.60
SalC	20.20	1.26	6.23
SalD	4.18	0.39	9.22

Table 9 - Inter-day Precision (n=3)

Compound	Mean (mg/g)	Standard Deviation (mg/g)	Coefficient of Variation(%)
SalA	758.22	50.32	6.64
SalB	283.96	42.26	14.88
SalC	18.70	2.58	13.82
SalD	3.51	0.64	18.22

3.2.3- Sensitivity

In the method validation of the present thesis, a signal-to-noise ratio of 3 was implemented to measure LOD. Salvinorin A concentration of 1.25 µg/mL was determined as LOD (Table 10; Fig. 20).

Regarding LLOQ, a signal-to-noise ratio of 10 was studied. Concentration of 2.5 µg/mL was determined as LLOQ (Table 10; Fig. 20).

Table 10 - LOD and LLOQ for salvinorin A

Concentration	*Mean of Peak Areas*	*Standard Deviation*	*Coefficient of Variation*	*S/N ratio*
1,25 µg/mL	*7.35E+05*	*1.01E+05*	*13.73%*	*4*
2,5 µg/mL	*1.20E+06*	*7.06E+04*	*5.90%*	*15*

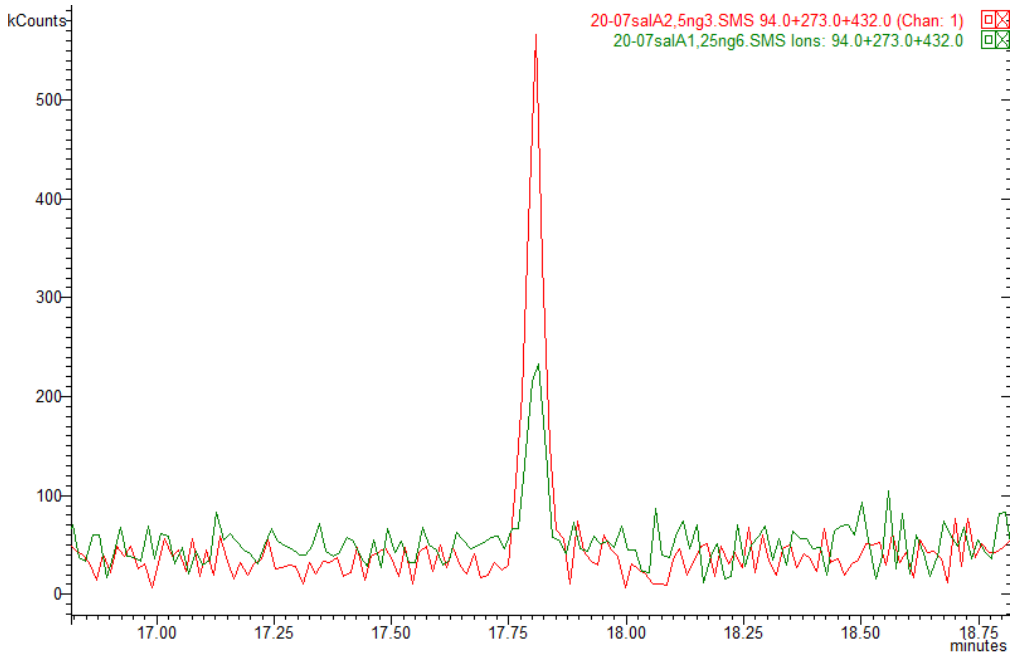


Fig. 20 - Overlaped peaks for LOD (green line) and LLOQ (red line) concentrations

3.3- Salvinorins identification

3.3.1- Salvinorin A detection

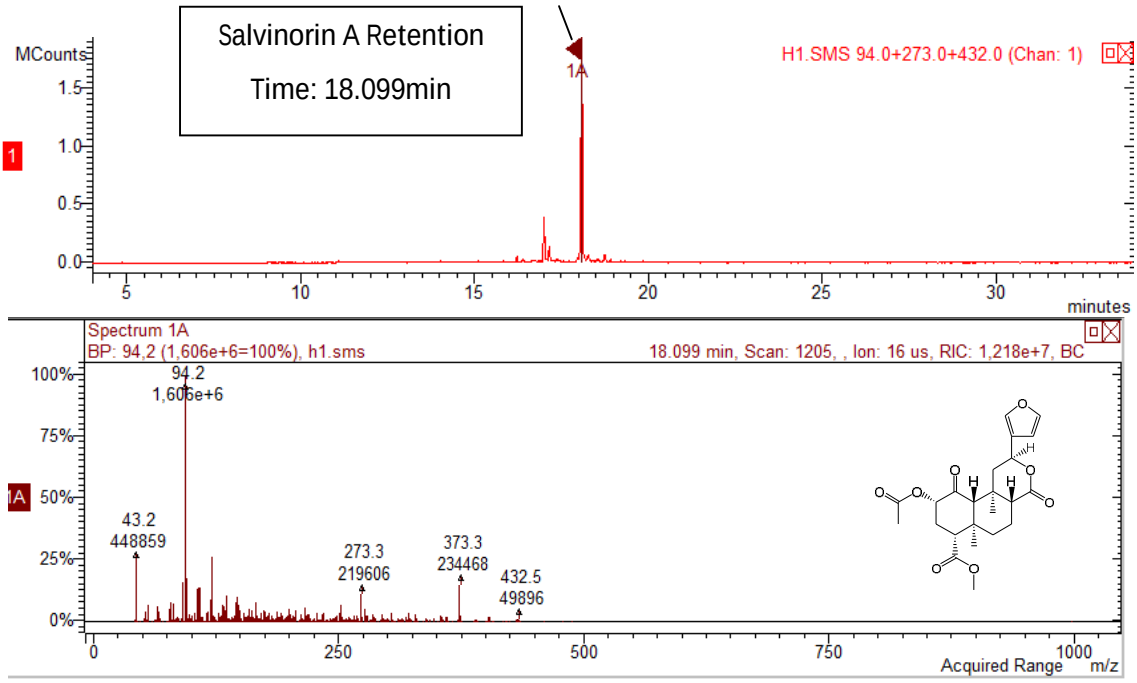


Fig. 21 **Full scan reconstructed chromatogram of an acetonitrile sample extract using m/z 94, m/z 273, m/z 432, Salvinorin A characteristic m/z ions.**

The selected ions used for the detection of salvinorin A were: m/z 94, m/z 273 and m/z 432. In the chromatogram showed in figure 21, the retention time of the compound was 18.099 minutes.

SWG DRUG library of spectra matched 80,4%, as the probability of being salvinorin A. Salvinorin A was the most abundant salvinorin in all samples.

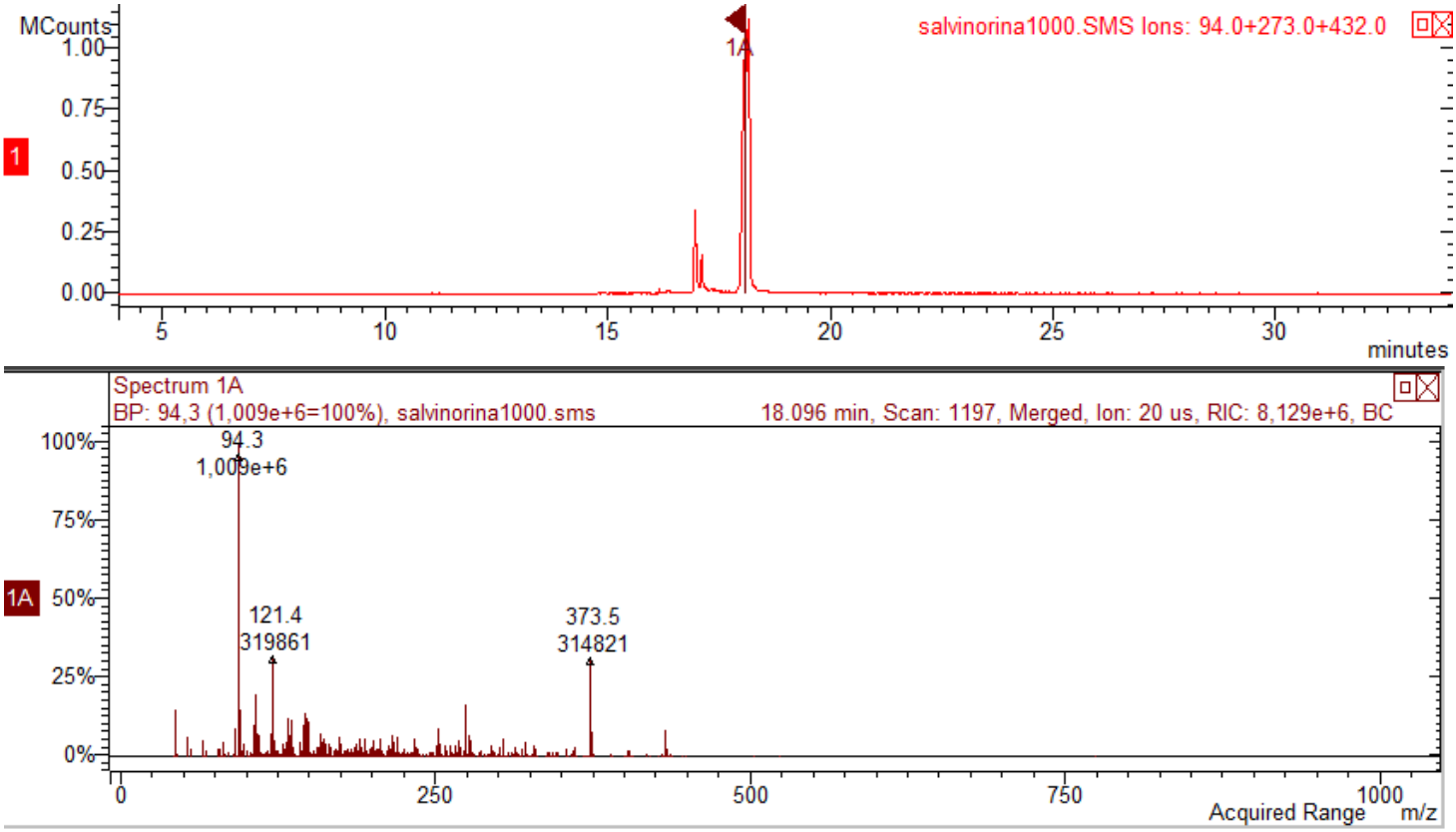


Fig. 22 – Full scan reconstructed chromatogram of salvinorin A standard using m/z 94, m/z 273, m/z 432 Salvinorin A characteristic m/z ions.

Salvinorin A standard revealed an identical mass spectrometry profile of the one present in samples. The retention time was also similar: 18,169 min (Fig. 22).

3.3.2 – Salvinorin B detection

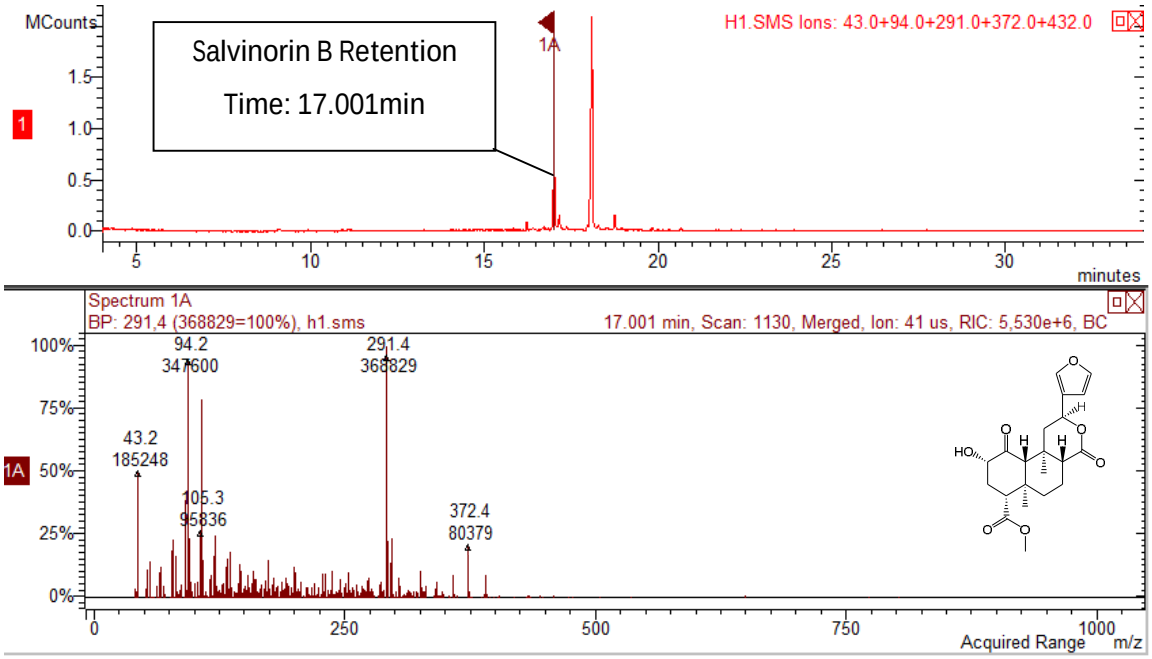


Fig. 23 - Full scan reconstructed chromatogram of an acetonitrile sample extract using m/z 43, m/z 94, m/z 291, m/z 372, m/z 432, Salvinorin B characteristic m/z ions.

The selected ions used for the detection of salvinorin B were: m/z 43, m/z 94, m/z 291 m/z 372 and m/z 390. In the chromatogram above exhibited, the retention time of the compound was 17.001 minutes, which was similar in other studied samples. As evidenced in Fig.24, salvinorin B had the lowest retention time of all salvinorins, therefore eluting in first place.

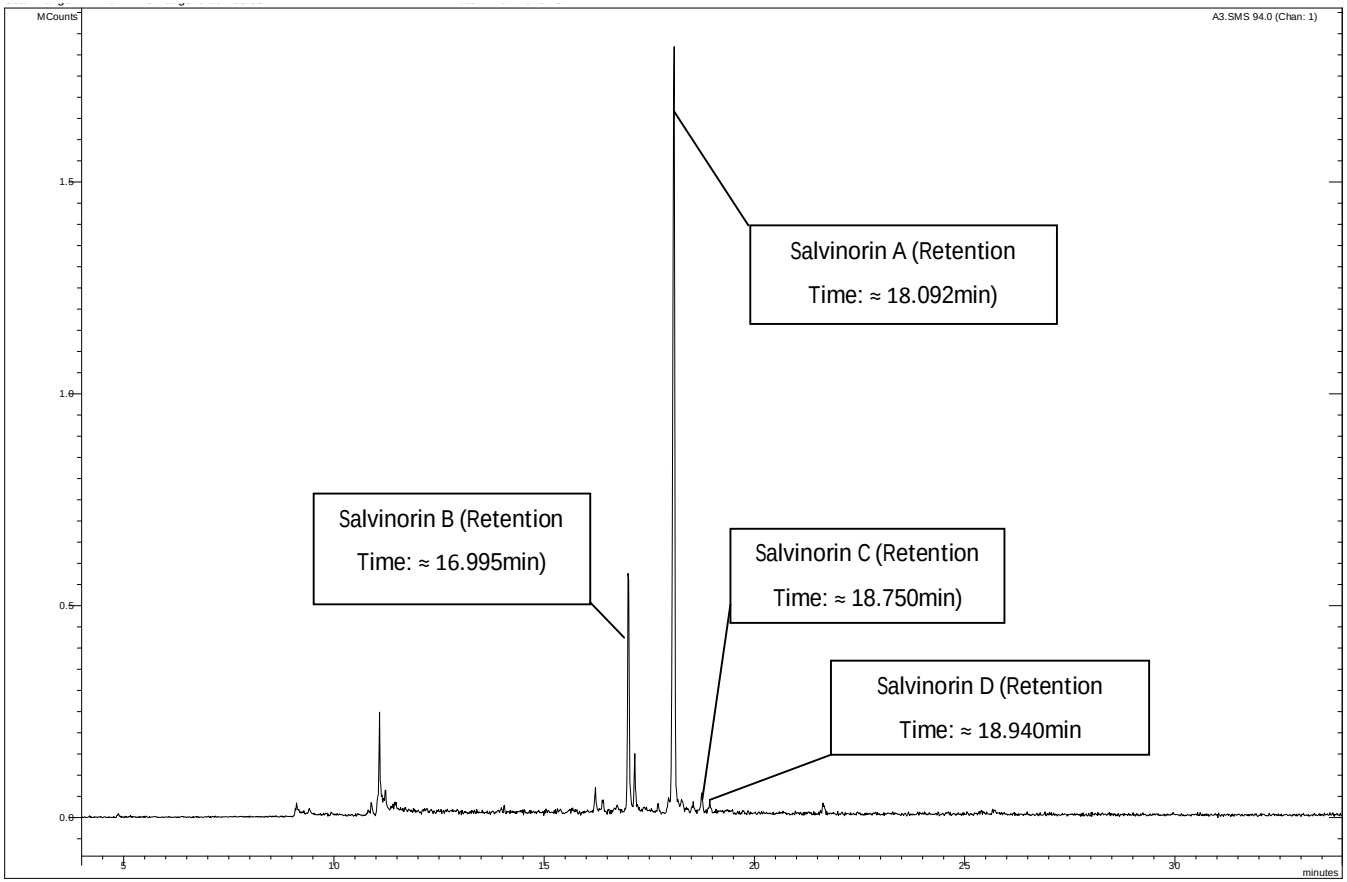


Fig. 24 – Full scan chromatogram of a *Salvia divinorum* acetonitrile concentrated extract

3.3.3 – Salvinorin C detection

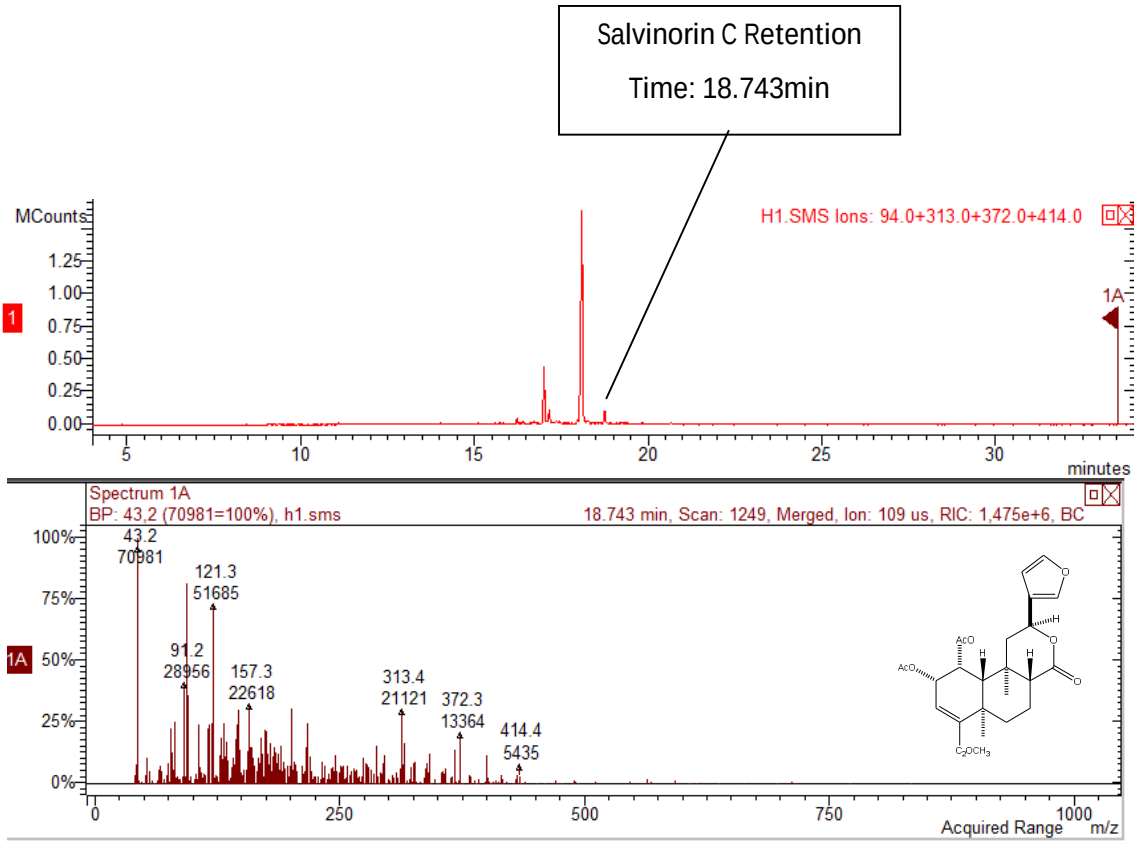


Fig. 25 - Full scan reconstructed chromatogram of an acetonitrile sample extract using m/z 94, m/z 313, m/z 372, m/z 414 Salvinorin C characteristic m/z ions.

The selected ions used for the detection of salvinorin C were: m/z 94, m/z 313 m/z 372 and m/z 414. In the chromatogram above exhibited, the retention time of the compound was 18.743 minutes.

3.3.4 – Salvinorin D detection

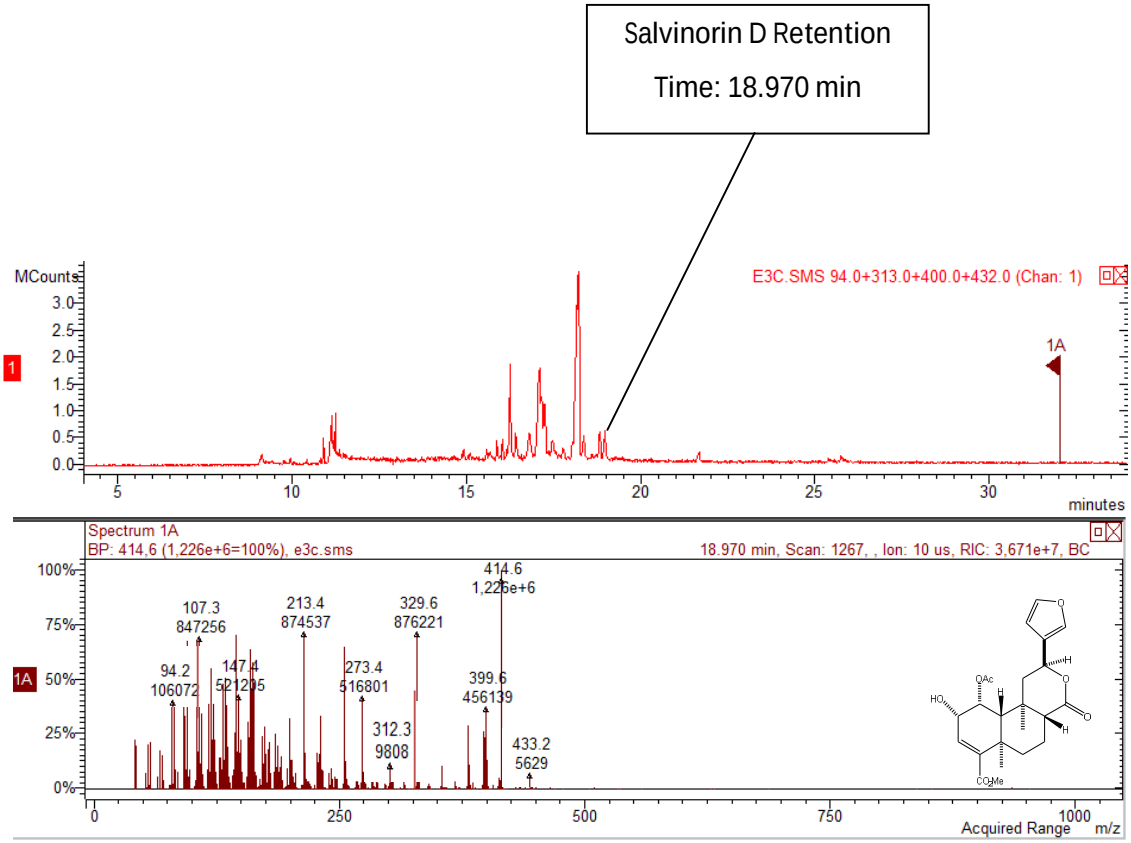


Fig. 26 - Full scan reconstructed chromatogram of an acetonitrile sample extract using m/z 94, m/z 313, m/z 400, m/z 432 Salvinorin D characteristic m/z ions.

The selected ions used for the detection of salvinorin D were: m/z 94, m/z 313, m/z 400 and m/z 432. In the chromatogram above exhibited, the retention time of the compound was 18.970 minutes. The identification of salvinorin D was very difficult because of the low concentration of the compound, and also because of the similar retention time of other compound, estimated to be β-sitosterol. The mass spectrum contemplates the characteristic m/z ions of β-sitosterol (such as m/z 414) (Huang et al., 2007). REPLIB library of spectra matched 46.2%, as the probability of being β-sitosterol.

3.4- Quantification of salvinorins in the concentrated extracts

3.4.1- Quantification of salvinorin A

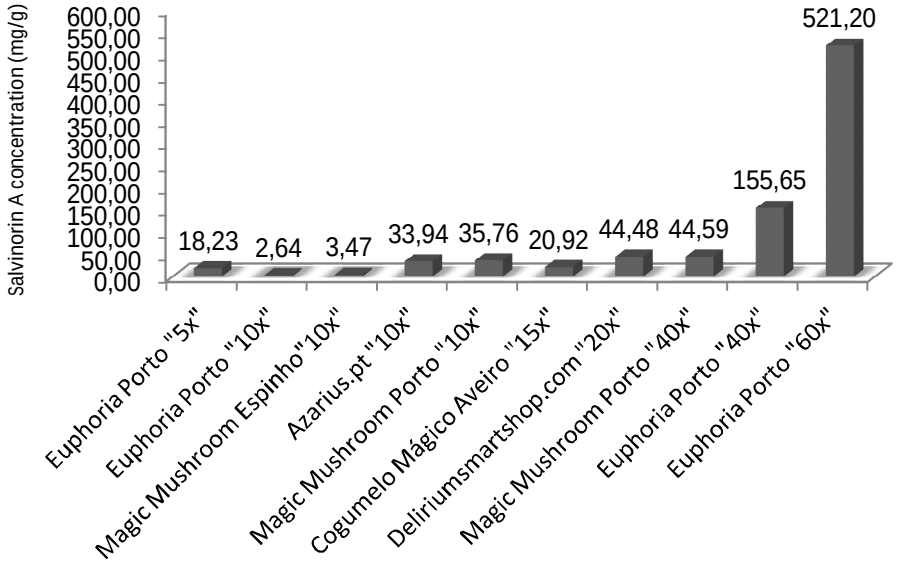


Fig. 27 - Concentrations of salvinorin A in samples

In most of the cases, the products that had higher labeled potencies had higher concentrations of salvinorin A (Table 9). However, there were some exceptions (Fig. 27; Table 11).

Table 11 - Salvinorin A concentration in different samples

Samples	Salvinorin A concentration (mg/g)
Euphoria Porto "5x"	*18.23*
Euphoria Porto "10x"	*2.64*
Magic Mushroom Espinho"10x"	*3.47*
Azarius.pt "10x"	*33.94*
Magic Mushroom Porto "10x"	*35.76*
Cogumelo Mágico Aveiro "15x"	*20.92*
Deliriumsmartshop.com "20x"	*44.48*
Magic Mushroom Porto "40x"	*44.59*
Euphoria Porto "40x"	*155.65*
Euphoria Porto "60x"	*521.20*

3.4.2- Quantification of salvinorin B

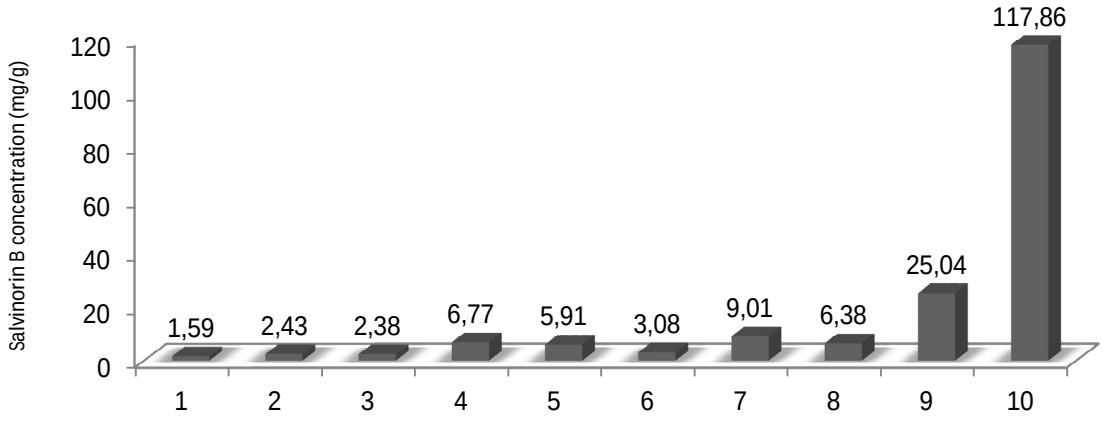


Fig. 28 - Concentration of salvinorin B in different samples

Salvinorin B, already identified as the most important metabolite of salvinorin A (McDonough et al., 2008; Schmidt et al., 2005), and the second most prevalent compound in *Salvia divinorum* samples, had, in the present work, concentrations directly proportional to salvinorin A concentrations.

Table 12 - Salvinorin B Concentration in different samples

Samples	Salvinorin B concentration (mg/g)
Euphoria Porto "5x"	*2.38*
Euphoria Porto "10x"	*1.59*
Magic Mushroom Espinho"10x"	*2.43*
Azarius.pt "10x"	*6.77*
Magic Mushroom Porto "10x"	*5.91*
Cogumelo Mágico Aveiro "15x"	*3.08*
Deliriumsmartshop.com "20x"	*9.01*
Magic Mushroom Porto "40x"	*6.38*
Euphoria Porto "40x"	*25.04*
Euphoria Porto "60x"	*117.86*

In order to study the relationship between the concentration of salvinorin A and salvinorin B, a model of linear correlation was studied (Fig. 29). The study tested the possibility of salvinorin B concentration be dependent on salvinorin A concentration.

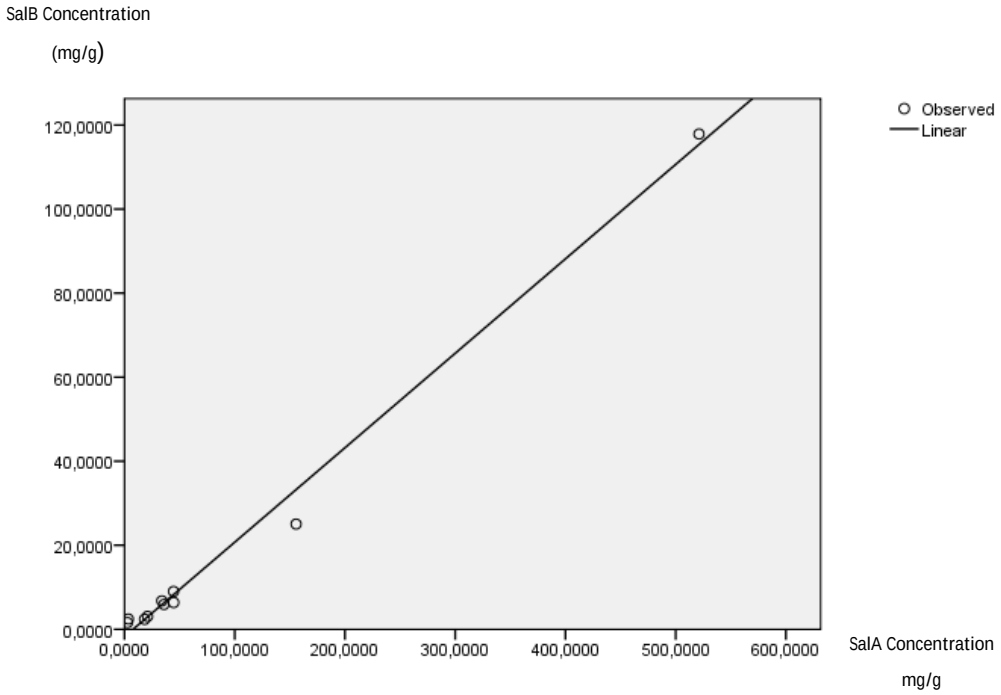


Fig. 29 - Linear Correlation Between Concentrations of Salvinorins A and B

To evidence a statistically significant relation between the concentrations of both compounds, a test of hypothesis was also made. In this test, H_0 represents the hypothesis of no linear correlation (H_0: $\beta_1 = 0$).

Table 13 - SPSS output, on test of linear correlation between salvinorins A and B

Coefficients[a]

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
1(Constant)	-1.751	1.279		-1.369	.208
SalA	.225	.007	.996	30.584	.000

a. Dependent Variable: SalB

p-value

The SPSS 16.0 output provides a p-value (0.000) below the level of significance adopted (0.05) (Table 13). Therefore, it might be affirmed that the independent variable (salvinorin A) has statistically significant power to predict the dependent variable (salvinorin B), since it can be rejected the null hypothesis. The equation that traduces this relationship is: y= -1.751 + 0.225x.

It was also possible to estimate the determination coefficient. According to the following SPSS 16.0 output, the adjusted R square possesses a value of 0.99 (Table 14). This means that, the independent variable average explains 99% of the variation of the dependent variable.

Table 14 - SPSS output on prediction of salvinorin B concentration from salvinorin A concentration

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
	.996[a]	.992	.990	3.4895883

a. Predictors: (Constant), SalA

3.4.3- Quantification of salvinorin C

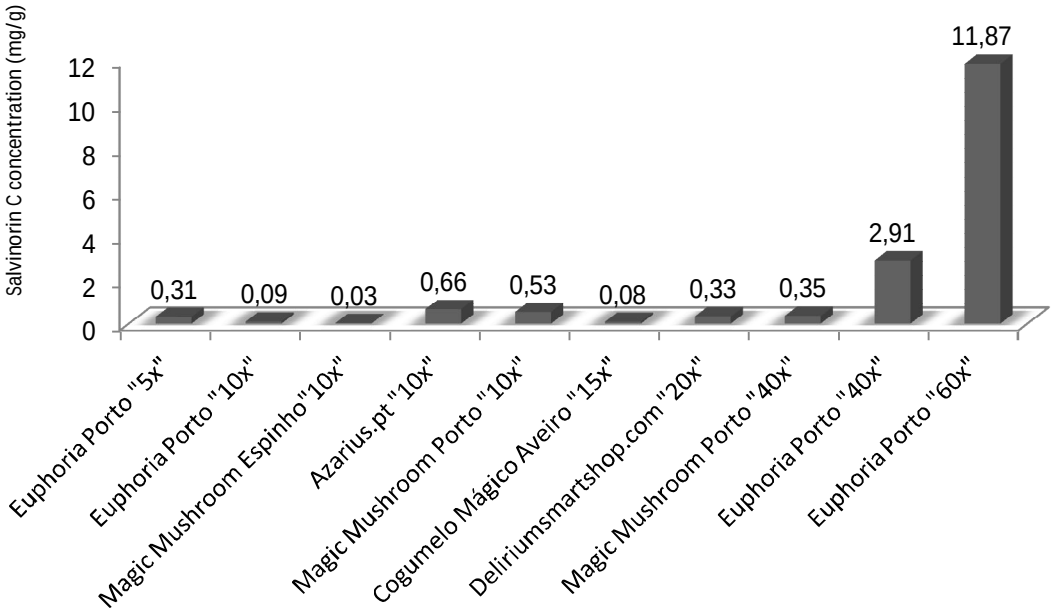


Fig. 30 - Salvinorin C concentrations in different samples

Salvinorin C concentration in samples was also directly proportional to salvinorin A concentration. Salvinorin C was the third most prevalent compound in the studied samples, after salvinorins A and B.

Table 15 - Salvinorin C Concentrations in different Samples

Samples	Salvinorin C concentration (mg/g)
Euphoria Porto "5x"	*0.31*
Euphoria Porto "10x"	*0.09*
Magic Mushroom Espinho"10x"	*0.03*
Azarius.pt "10x"	*0.66*
Magic Mushroom Porto "10x"	*0.53*
Cogumelo Mágico Aveiro "15x"	*0.08*
Deliriumsmartshop.com "20x"	*0.33*
Magic Mushroom Porto "40x"	*0.35*
Euphoria Porto "40x"	*2.91*
Euphoria Porto "60x"	*11.87*

In order to study the relationship between the concentration of salvinorin A and salvinorin C, a model of linear correlation was studied, equal to the model applied to the study of the relationship between salvinorins A and B (Fig. 31). The study also tested the possibility of salvinorin C has its concentration dependent on salvinorin A.

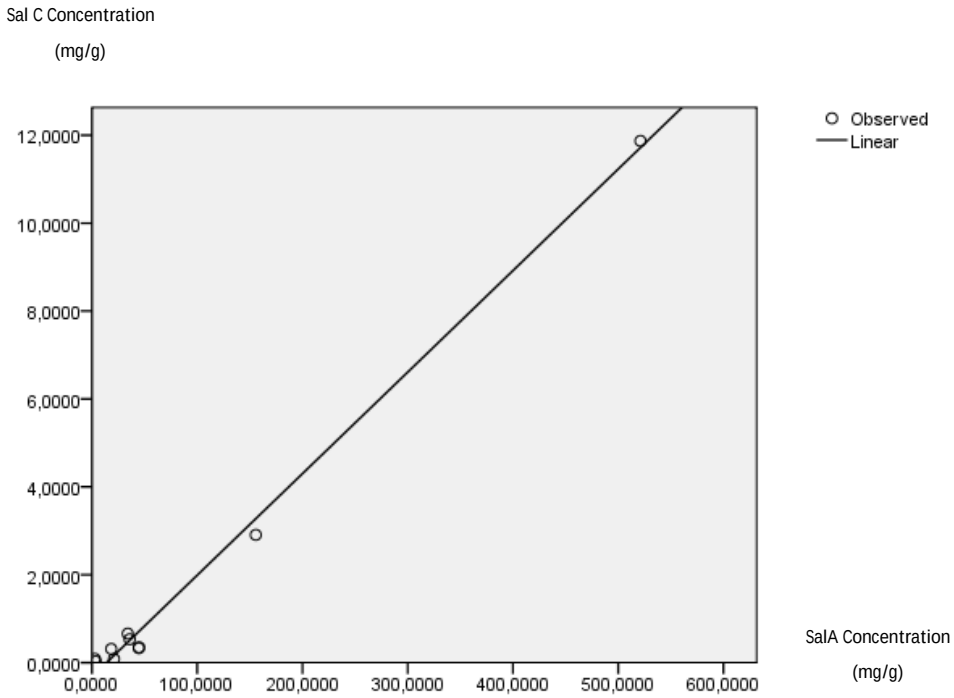


Fig. 31 - Linear Correlation Between Concentrations of salvinorins A and C

A test of hypothesis was made, to evidence a statistically significant relation between the concentrations of both of the compounds, similar to the previous one applied to the relationship between salvinorins A and B (H_0: $\beta_1 = 0$).

Table 16 - Table 9 - SPSS output on test of linear correlation between Salvinorins A and C

Coefficients[a]

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	-.319	.109		-2.918	.019
SalA	.023	.001	.997	36.763	.000

a. Dependent Variable: SalC

p-value

The SPSS 16.0 output provides a p-value (0.000) below the level of significance adopted (0.05) (Table 16). Therefore, it might be affirmed that the independent variable (salvinorin A) has statistically significant power to predict the dependent variable (salvinorin C), since it can be rejected the null hypothesis. The equation that traduces this relationship is: y= -0.319 + 0.023x.

It was also possible to estimate the determination coefficient. According to the following SPSS 16.0 output, the adjusted R square possesses a value of 0.993 (Table 17). This means that, the independent variable average explains 99.3% of the variation of the dependent variable.

Table 17 - SPSS output on Prediction of salvinorin A concentration over salvinorin C concentration

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
	.997[a]	.994	.993	.2983484

a. Predictors: (Constant), SalA

3.4.4- Quantification of salvinorin D

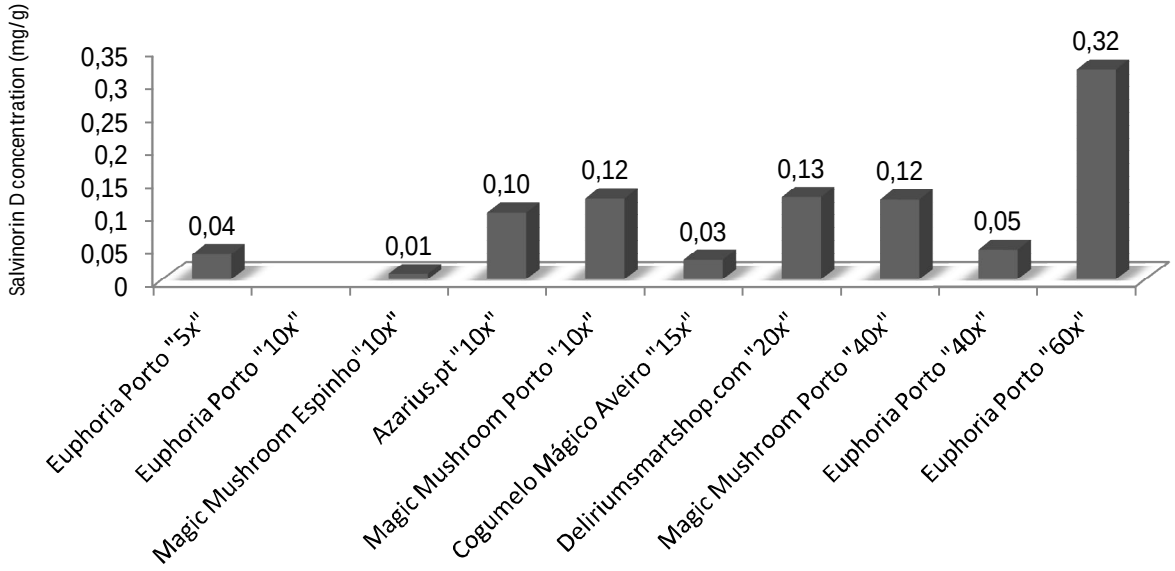


Fig. 32 - Salvinorin D Concentrations in different samples

Among all salvinorins detected, salvinorin D was the least representative. Most of the samples had very small concentrations of salvinorin D, and in one of the samples it was not possible to detect and quantify it (should be remembered that the determined LOD and LLOQ of salvinorin A standard were respectively 1.25µg/mL and 2.5 µg/mL) (Table 18).

Table 18 - Salvinorin D Concentrations in different Samples

Samples	Salvinorin D concentration (mg/g)
Euphoria Porto "5x"	*0.04*
Euphoria Porto "10x"	*Not determined*
Magic Mushroom Espinho"10x"	*0.01*
Azarius.pt "10x"	*0.10*
Magic Mushroom Porto "10x"	*0.12*
Cogumelo Mágico Aveiro "15x"	*0.03*
Deliriumsmartshop.com "20x"	*0.13*
Magic Mushroom Porto "40x"	*0.12*
Euphoria Porto "40x"	*0.05*
Euphoria Porto "60x"	*0.32*

3.5- Comparison with previous studies

As long as we know, the literature presently reports two scientific studies concerning the identification and quantification of salvinorin A in concentrated extracts of *Salvia divinorum*. These works from Wolowich et al. (2006) and Tsujikawa et al. (2008) only contemplated samples with labeled potencies ranging from "1x" to "20x" and from "2x" to "25x". Our study was the first to analyze commercialized products with labeled potencies as high as "40x" and "60x".

Although the labeled potencies of the purchased products in both mentioned studies are approximately in the same range, the results obtained were different. While Wolowich et al. (2006) presented a range concentration of salvinorin A between 0.126 – 0.951 mg/g, Tsujikawa et al. (2008) presented a range concentration between 4.1-38.9 (mg/g).

In order to compare the range of concentrations with both mentioned published works, it was only taken into account the seven samples in which the labeled potency was between "5x" and "20x". The obtained range is similar to the one presented by Tsujikawa et al. (2008): 2.6 mg/g and 44.5 mg/g, thus, quite different from the other study (Table 19).

Table 19 - Range of Concentrations of Salvinorins A and B in different researchs about concentrated extracts of *Salvia divinorum*

Study (Considering samples with labeled potency between "2X" and "25X")	Range of concentrations of Salvinorin A (mg/g)	Range of concentration of Salvinorin B(mg/g)
Wolowich et al. (2006) ("1X"-"20X") (n=5)	0.126 – 0.951	Not determined
Tsujikawa et al. (2008) ("2X"-"25X") (n=9)	4.1 – 38.9	0.26 – 2.4
Present Study ("5X"-"20X") (n=10)	2.6 – 44.5	1.59 – 9.01

To confirm the similarity of the results obtained by Tsujikawa *et al.* with the results obtained in our study, it was determined the concentration per unit of labeled potency ((mg/g) / "x"potency) of each sample (Table 20).

Table 20 - Concentration of salvinorin A per unit of labeled potency in all samples from Tsujikawa et al., 2008 and samples from the present study

Samples (Present Study)	Concentration per unit of labeled potency ((mg/g)/"x" potency)	Samples (Tsujikawa et al., 2008)	Concentration per unit of labeled potency ((mg/g)/"x" potency) - Tsujikawa *et al.*
Euphoria Porto "5x"	*3.65*	***2x***	*2.05*
Euphoria Porto "10x"	*0.26*	***7x***	*0.94*
Magic Mushroom Espinho"10x"	*0.35*	***10x (A)***	*1.17*
Azarius.pt "10x"	*3.39*	***10x (B)***	*1.00*
Magic Mushroom Porto "10x"	*3.58*	***10x (C)***	*1.27*
Cogumelo Mágico Aveiro "15x"	*1.39*	***14x***	*1.11*
Deliriumsmartshop.com "20x"	*2.22*	***20x***	*1.37*
Magic Mushroom Porto "40x"	*1.11*	***20x***	*1.03*
Euphoria Porto "40x"	*3.89*	***25x***	*1.56*
Euphoria Porto "60x"	*8.69*	***-***	*-*

Student's t-test for independent samples was applied to evaluate if there was statistically significant evidence to reject the hypothesis of equality between two groups of samples (G1 corresponding to the present study, and G2 corresponding to Tsujikawa et al. (2008)) (Table 21).

Table 21 - SPSS output on Student's t-test for independent samples

Autor	N	Mean	Std. Deviation	Std. Error Mean
G1	10	2.8539	2.47395	.78233
G2	9	1.2762	.34917	.11639

Table 22 - SPSS output on Student's t-test for independent samples

		Levene's Test for Equality of Variances		t- test for Equality of Means						
									95% Confidence Interval of Difference	
		F	Sig.	t	df	Sig (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Valores	Equal variances assumed	7.996	.012	1.891	17	.076	1.57767	.83436	-.18269	3.33802
	Equal variances not assumed			1.995	9.398	.076	1.57767	.79094	-.20010	3.35543

According to the outputs obtained in SPSS 16.0, for a 95% Confidence Interval of Difference, the value "0" is in the interval [-0.18269; 3.33802], therefore, the hypothesis (H_0) of equality between the results obtained, cannot be ruled out (Table 22).

As to salvinorin B, the values obtained by Tsujikawa *et al.*, ranged from 0.26 to 2.4 mg/g. The present study recorded concentrations of the same compound, in similar potency labeled products (5x-20x), between a minimum of 1.59 and a maximum of 9.01 mg/g. Since it was not possible to acquire the standard of salvinorin B, its concentration was estimated adapting the calibration curve of salvinorin A, which may have associated errors.

3.6- Comparison between the real concentration of salvinorin A and the the salvinorin A labeled in the marketed package

Most of the purchased samples did not provide information about the concentration of salvinorin A, only referring the "potency" of the products. Azarius® website was the only manufacturer that indicated salvinorin A content. The sample from Azarius® ("10X") had, according to the company's website, 25 mg of salvinorin A. After the analysis of the sample, it was estimated a concentration of 34 mg of salvinorin A. Therefore, in this case, the marketer sustained a concentration 26% under its real concentration (Table 23). This misleading information might eventually cause intoxications, overdoses or tolerance towards this drug.

Table 23 - Comparison of the amount of salvinorin A publicized by Azarius, and the amount experimentally determined

Concentration of salvinorin A in Azarius® sample (10X) – Obtained result after analysis	Concentration of salvinorin A in Azarius® sample (10X) – According to company's website
34mg	25mg **(-26%)**

The other marketer indicating the contents in salvinorin A, was Euphoria®. However, in this case, the mass of salvinorin A was not objectively mentioned. Instead, the company ensures that 1 g of "*Salvia divinorum* 5x" corresponds to 5 g of *Salvia divinorum* leaves; 1 g of "*Salvia divinorum* 10x" corresponds to 10 g of *Salvia divinorum* leaves; 1 g of "*Salvia divinorum* 40x" corresponds to 40 g of *Salvia divinorum* leaves; and 1 g of "*Salvia divinorum* 60x" corresponds to 60 g of *Salvia divinorum* leaves (Fig. 33).

In accordance to Medana et al. (2006) studies, the amount of salvinorin A in leaves of *Salvia divinorum* originating from Sierra Mazatec is 0.76% (w/w), while the amount of salvinorin A in *Salvia divinorum* leaves originating from Hawaii is 0.78% (w/w). On the other hand, Kennedy and Wiseman (2010) ensured the extraction of 0.80% (w/w) of salvinorin A from *Salvia divinorum* leaves.

In order to clarify the reliability of information provided by Euphoria®, all samples, with the exception of the one from Azarius®, were submitted to comparison between the experimentally determined concentrations of salvinorin A and the concentrations presumably advertised by the manufacturers. The percentages of the difference of the expected results and the ones obtained experimentally were determined (Table 24).

The majority of samples had a concentration much lower than the expected, including 3 out of 4 samples from Euphoria® (less 95%-2567% of the advertised concentration). Only the highest labeled potency sample ("60X") from Euphoria® had an experimentally observed concentration approximate to the expected one (8%-12% above the expected).

Besides the lack of information about salvinorin A concentration, several samples revealed insufficient information about other items as batches (not mentioned in 3 samples) and expiration date (not mentioned in 2 samples, and with two different expiration dates in one sample). Some samples provide scarce information about how to use the drug (only stating that must be smoked in a pipe or bong, and sometimes explaining that it must be used to produce an infusion). Surprisingly, the information most often exhibited in the packages were the expressions "Do not cause dependence" and "Not dangerous" (both mentioned in 6 samples).

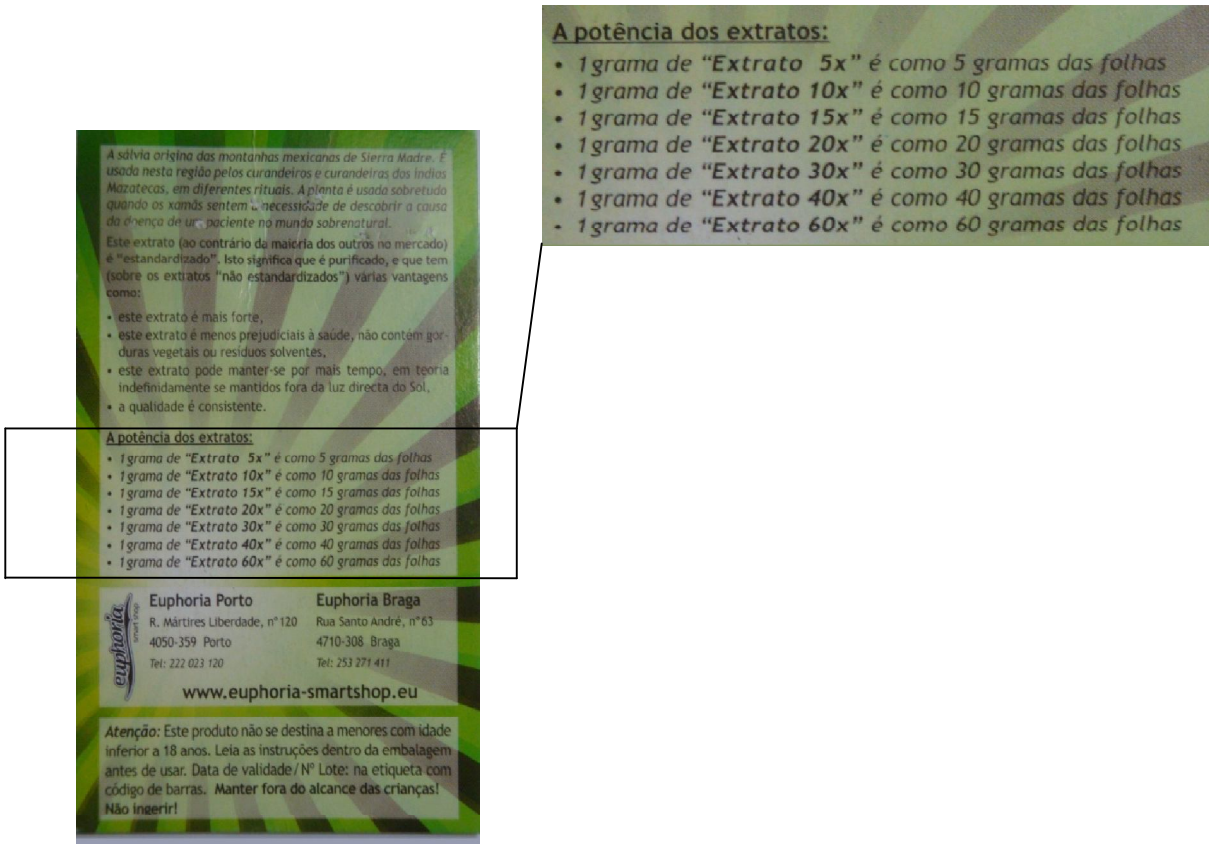


Fig. 33 - Concentration of the extracts, according to Euphoria

Samples	Real concentration of salvinorin A per (mg/g)	Expected concentration of salvinorin A (mg/g) (*Salvia divinorum* originated from Sierra Mazateca) - (Medana et al., 2006)	Expected concentration of salvinorin A (mg/g), (*Salvia divinorum* originated from Hawaii) (mg) - (Medana et al., 2006)	Expected concentration of salvinorin A (mg/g) per package (Kennedy & Wiseman, 2010)
Euphoria Porto "5x"	**18**	38 (+111%)	39 (+116%)	40 (+122%)
Euphoria Porto "10x"	**3**	76 (+2433%)	78 (+2500%)	80 (2567%)
Azarius.pt "10X"	**34**	76 (+124%)	78 (+129%)	80 (+135%)
Magic Mushroom Porto "10x"	**36**	76 (+111%)	78 (+116%)	80 (+122%)
Magic Mushroom Espinho "10x"	**3**	76 (+2433%)	78 (+2500%)	80 (+2567%)
Cogumelo Mágico Aveiro "15x"	**42**	228 (+443%)	234 (+457%)	240 (+471%)
Deliriumsmartshop.com "20x"	**44**	152 (+245%)	156 (+254%)	160 (+264%)
Magic Mushroom Porto "40x"	**45**	304 (+576%)	312 (+594%)	320 (+611%)
Euphoria Porto "40x"	**156**	304 (+95%)	312 (+100%)	320 (+105%)
Euphoria Porto "60x"	**521**	456 (-12%)	468 (-10%)	480 (-8%)

Table 24 - Comparison of the amount of salvinorin A estimated by Euphoria and the amounts determined by previous scientific works

3.7- Amount known to induce hallucinogenic effects, in each sample

The companies that sell *Salvia divinorum* concentrated extracts are not very accurate about the amount that must be smoked. Marketers usually advice to smoke a *"small amount"* of the content of the purchased product, regardless the labeled potency of the extract.

According to Siebert (1994), 200 µg of salvinorin A are generally enough to obtain hallucinogenic effects.

Given the concentration of each sample, it was estimated the amount of the concentrated extract that was needed to obtain the pretended effects (Table 25).

Table 25 - Amount of Salvia known to induce hallucinogenic effects, per sample

Samples	Salvinorin A Concentration (mg/g)	Amount of *Salvia* known to induce hallucinogenic effects (mg)
Euphoria Porto "5x"	*18.2*	*11.0*
Euphoria Porto "10x"	*2.6*	*75.8*
Magic Mushroom Espinho"10x"	*3.5*	*57.7*
Azarius.pt "10x"	*33.9*	*5.9*
Magic Mushroom Porto "10x"	*35.8*	*5.6*
Cogumelo Mágico Aveiro "15x"	*20.9*	*9.6*
Deliriumsmartshop.com "20x"	*44.5*	*4.5*
Magic Mushroom Porto "40x"	*44.6*	*4.5*
Euphoria Porto "40x"	*155.6*	*1.3*
Euphoria Porto "60x"	*521.2*	*0.4*

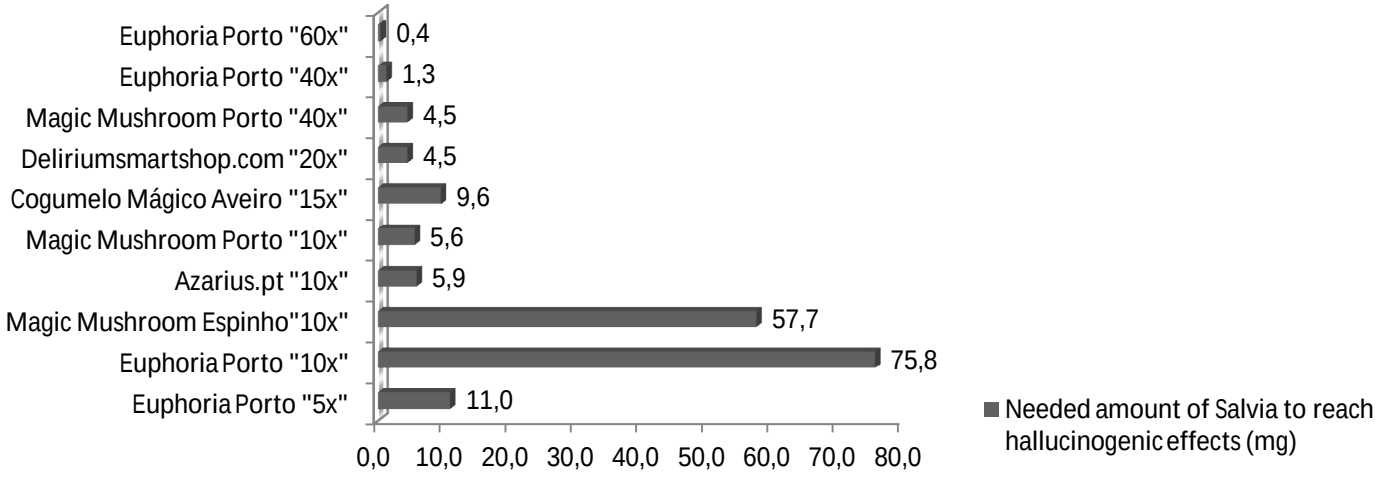


Fig. 34 - Needed amount (mg) of Salvia concentrated extract to reach hallucinogenic effects

In the studied samples the amount known to induce hallucinogenic effects ranged from 0.4 mg to 75.8 mg (Fig. 34). These results reveal, in all analyzed samples, that the consumption of a very small amount of the extract is able to produce hallucinogenic effects. Since most of the consumers do not have this knowledge when buying *Salvia divinorum* extracts with the highest labeled potencies, they are consuming excessive amounts of the drug that might eventually be extremely dangerous.

3.8- Critical analysis of prices of the concentrated extracts

In order to evaluate the prices of the purchased products, two different approaches were made: the determination of a relationship between the price of the products and their labeled potency, and the association between the price and the salvinorin A concentration in samples.

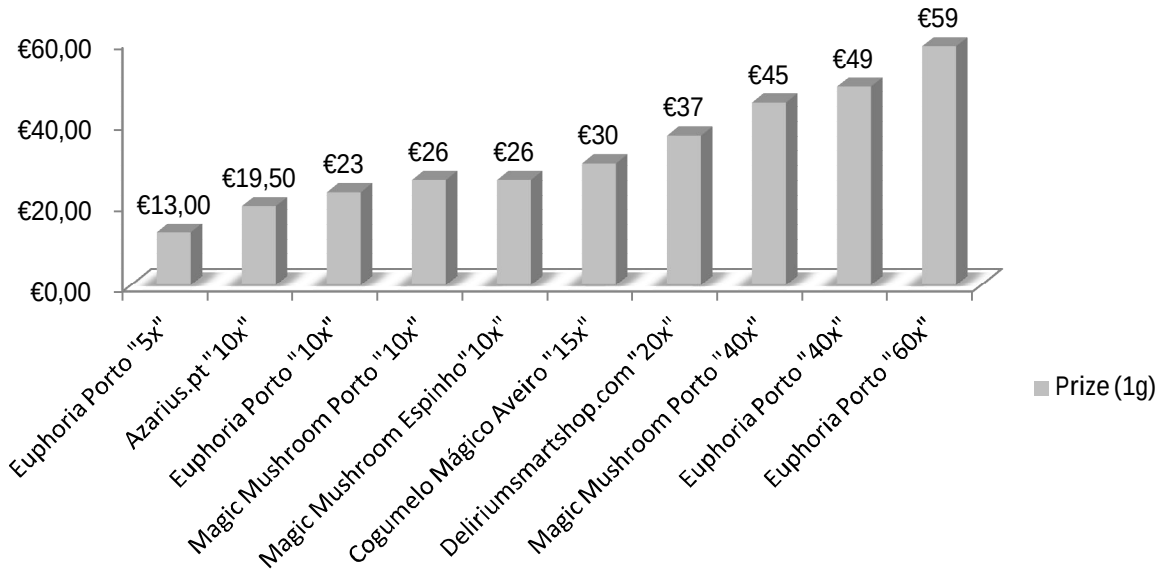


Fig. 35 - Relationship between price and labeled potency

According to the results presented in Fig 35, there is a relationship between commercial prices and the labeled potency (Fig.35). Nevertheless, since the concentration in salvinorin A is not always in accordance with the labeled potency, this relation between the price and salvinorin A concentration is not proportional (Fig.36).

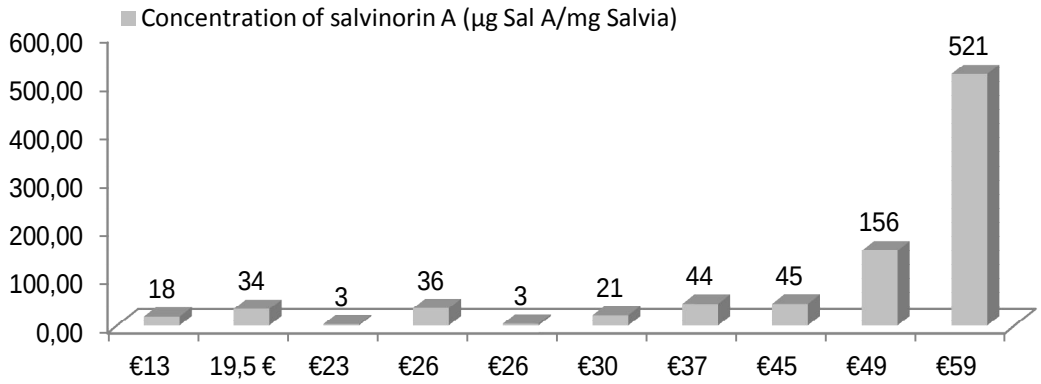


Fig. 36 - Relationship between price and salvinorin A concentration

In some cases, buying products with a concentration of 18 mg/g costs half of the price of a product with a concentration of approximately 3 mg/g. These results suggest that some commercialized products might not correspond to the expectations of the consumers.

4- Conclusion

A methodology using GC/MS analysis was developed to determine four important salvinorins (A, B, C, D) in ten *Salvia divinorum* commercialized products. The method developed showed a correlation coefficient of linearity of 0.9951 for salvinorin A, an intra-day precision between 3.63% and 9.22% and an inter-day precision between 6.64% and 18.22%. The LOD was 1.25 µg/mL and the LLOQ was 2.5 µg/mL.

The concentrations of salvinorin A in the studied samples are, in some cases, much higher than the needed to obtain hallucinogenic effects, implying a meticulous weighing of the amount to be consumed, which certainly will not be done by consumers. Besides, the labeled potencies do not match the real concentrations of salvinorin A. These facts might lead to eventual intoxications, overdoses or tolerance towards this drug.

In conclusion, concerns about the sale of *Salvia divinorum* products either it is being considered legal or illegal must be reinforced. The analysis of concentrated extracts of *Salvia divinorum*, from different marketers, allowed the identification of four salvinorins, salvinorin A being the most prevalent. The presence of this compound ensures the hallucinogenic properties stated by the sellers, but there are several unreliable data provided to consumers that might be worrying. Most of the time, there is no information on salvinorin A concentration, but when it is available, generally does not correspond to the true amount present in products. Besides, the labeled potency that is always present in packages not always corresponds to a direct proportional concentration of salvinorin A, as expected.

Part III:

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